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THYROTROPIN ASSAY BASED ON THE DEPLETION OF IODINE IN CHICK THYROIDS

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THYROTROPIN assays have been based on either the secondary effects due to the increased activity of the stimulated thyroids or on one or more of the specific effects induced on the thyroid gland by the TSH stimulation of the glandular secretory mechanism. The former assays have been predominantly based on the accelerated metamorphosis of the amphibian larvae (Cuyler *et al.*, 1936; D'Angelo and Gordon, 1950), while the latter assays have been based on the increase in thyroid weight (Rowlands and Parkes, 1934; Smelser, 1938; Bergman and Turner, 1939), on the increase in acinar cell height (Starr and Rawson, 1937; Rawson and Salter, 1940; Adams, 1942), on the appearance of a histological response (Jorgensen and Wade, 1941; Ciereszko, 1945), on the increase in the intracellular colloid droplets of the thyroid gland (Dvoskin, 1947) and on the decrease in the thyroid iodine content (Stimmel *et al.*, 1936; Cuyler *et al.*, 1936). Excellent reviews of the existing thyrotropin assay methods have been included in the articles by White (1944), Adams and Beeman (1946) and Albert (1949).

The method presented in this paper is based on the depletion of iodine in the thyroids of chicks. It is dependent on the comparison of the responses obtained from TSH preparations of unknown potencies with the responses elicited by 2–3 levels of a standard TSH preparation run concomitantly.

MATERIALS AND METHODS

White Leghorn cockerels (from several midwest hatcheries) 1–2 days of age, averaging 30–45 gm. in body weight were utilized. These chicks, kept in a brooder at a fairly

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[1] Deceased.

constant temperature, were maintained on a commercial chick starter mash (Purina).

Three different kinds of materials were used as reference TSH standards at the three stages of assay development. Initially, beef anterior pituitary acetone powder, BAP #1, was used. This was then superceded by 2R3 with an assumed TSH potency of 100%. It was a partially purified TSH obtained by alkaline-alcohol fractionation of a beef anterior pituitary acetone powder. Its hormonal contaminants included ACTH (.06 I.U. per mg. by the ascorbic acid depletion method), gonadotropin (approximately 6 Collip units per mg.), growth hormone (approximately 15% of pure beef growth hormone by the 10 day weight gain method in hypophysectomized rats), oxytocin (.03 U.S.P. units per mg.) and pressor (less than 0.03 U.S.P. units per mg.). Finally the U.S.P. Reference Thyrotropin replaced 2R3 as the standard. One U.S.P. thyrotropin unit is that amount of thyrotropin activity contained in 20 mg. of the U.S.P. Reference Substance; in terms of activity, 2.70 mg. of 2R3 is equivalent to one U.S.P. unit.

The samples containing unknown amounts of thyrotropin included 317-51 which was used in the preparation of the U.S.P. Thyrotropin Reference Substance, various TSH fractions, clinical FSH, ACTH, LH and growth fractions as indicated below.

Upon arrival, 100–200 chicks were individually marked with colored dyes, randomly distributed into ten groups of approximately equal size and injected subcutaneously with 0.2 ml. of an appropriate solution. The groups consisted of a control group injected with saline, 2–3 groups treated with 2–3 levels of the standard TSH BAP #1 (2R3 or U.S.P. Thyrotropin Reference Substance) and several groups treated with thyrotropin fractions of unknown potencies (1–2 levels each). The TSH samples were dissolved in saline at approximately pH 7.5 with the aid of 0.1 N NaOH and stored in a deep freeze between injections.

The chicks were injected once daily for three days. On the fourth day, food was withheld very early in the morning. Twenty-four hours after the last injection, the chicks were killed with ether. The thyroids were carefully dissected out, as described by Cope (1938), rapidly weighed to the nearest 0.1 mg. on a Roller-Smith torsion balance and placed in vials containing redistilled 95% ethanol. One to two pairs of these thyroids were stored in each vial so that each group had ten vials for ten iodine determinations. The thyroids were then assayed for the total iodine content according to the method of Walaszek-Piotrowski and Koch (1952), the results being calculated as micrograms of iodine per milligram of wet thyroid tissue.

The iodine responses elicited in the thyroids of the chicks injected with the TSH samples of unknown potencies were compared with the responses of the chicks treated with the reference TSH. Potency ratios and standard errors were calculated by means of the Irwin log dose method (1937). The potencies were reported as percentages of TSH activity of standard 2R3 or as U.S.P. units of TSH per mg. sample.

RESULTS

As the initial source of thyrotropin, beef anterior pituitary acetone powder, BAP #1, was utilized. For injection purposes, it was triturated with saline, brought up to a volume containing 50 mg. of total solids per ml., extracted at a pH of 7.5 for four hours with constant stirring at room temperature and centrifuged. The resultant supernatant containing 30 mg. of total protein per ml. was then used as a reference standard for the assay of TSH. The doses 1.1, 2.2 and 4.5 mg. of BAP #1 gave a graduated de-

crease of thyroid iodine such that, when the responses (μg. iodine per mg. thyroid) were plotted against the log doses, a straight line resulted. When the dosage of this pituitary standard TSH was extended to include up to 36 mg. of BAP #1, the iodine responses tended to plateau while the thyroid weight responses did not (see Fig. 1). Consequently, 1.1, 2.2 and 4.5 mg.

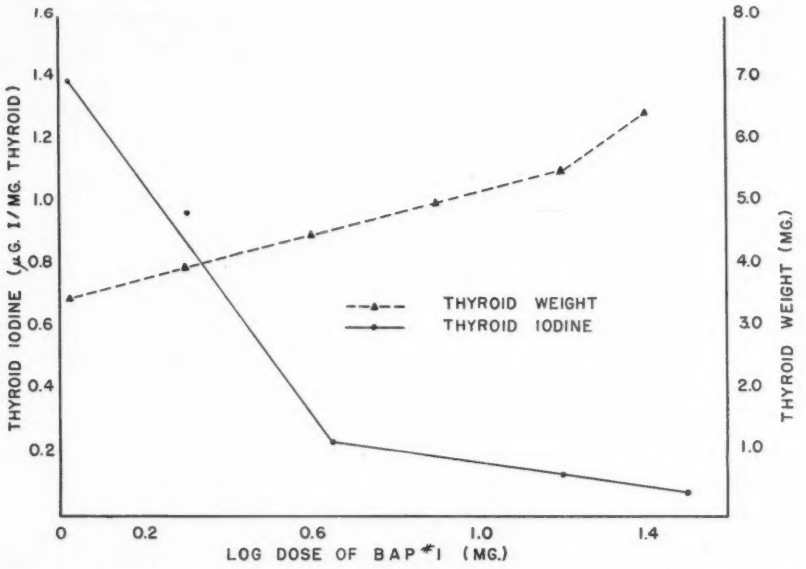

FIG. 1. Depletion of thyroidal iodine and increase in thyroid weight with acetone powder of beef anterior pituitary

of BAP #1 were used as a standard reference dose, and the thyroid iodine responses were used as the basis for TSH assay calculation.

As the fractionation of thyrotropin from beef anterior pituitary acetone powder proceeded, a large quantity of purer TSH fraction, 2R3, was obtained. It assayed $2100 \pm 600\%$ of BAP #1 and since it was only slightly contaminated with other pituitary hormones, it replaced BAP #1 as the reference standard. A total dose of 80 μg. of this TSH fraction gave a 30–50% decrease in thyroid iodine usually without any thyroid weight response. The total doses finally utilized were 25 μg., 50 μg., and 100 μg. or 40 μg., 80 μg., and 150 μg. A typical assay is shown in Table 1. The samples tested were various thyrotropin preparations with the exception of K45408R, a clinical FSH sample.

As a final step in this assay development, a U.S.P. Thyrotropin Reference Substance replaced the 2R3 as the reference standard. For purposes

TABLE 1. EXPERIMENTAL TSH ASSAY NO. 165

Control	Standard 2R3			TSH #3	TSH 2+3RW	K45408-R	317-51	U.S.P. TSH	
	0.040 mg.	0.080 mg.	0.160 mg.	0.080 mg.	0.080 mg.	0.530 mg.	0.032 mg.	0.016 u.	0.032 u.
Average Thyroid Weight (mg.)									
3.2 15*	3.2 14*	3.2 15*	3.7 15*	3.4 15*	3.7 13*	3.8 15*	3.2 15*	3.5 15*	3.5 15*
Thyroid Iodine Content (μg. I/mg. thyroid)									
1.64	1.43	1.12	0.58	1.28	0.95	0.63	1.01	1.39	0.90
1.33	1.38	0.88	0.44	1.03	0.76	0.52	0.98	1.18	0.81
1.21	1.17	0.81	0.44	0.82	0.66	0.44	0.93	1.16	0.66
1.20	0.92	0.73	0.42	0.71	0.60	0.38	0.57	0.97	0.61
0.97	0.83	0.68	0.41	0.63	0.41	0.35	0.54	0.91	0.60
0.91	0.80	0.57	0.34	0.61	0.40	0.33	0.51	0.69	0.42
0.88	0.74	0.49	0.32	0.57	0.39	0.32	0.48	0.54	0.41
0.81	0.73	0.44	0.31	0.54	0.35	0.22	0.40	0.41	0.28
0.77	0.68	0.39	0.17	0.40	0.32	0.21	0.37		0.27
0.70	0.60	0.23		0.36	0.25	0.15	0.34		
1.04	0.93	0.63	0.38	0.70	0.51	0.36	0.61	0.91	0.55

* Subscripts indicate number of animals per group.
Using Irwin's log dose method for calculation of TSH potencies, the results are:

TSH #3	88 ± 20% of Standard TSH 2R3
TSH 2+3RW	140 ± 33% of Standard TSH 2R3
K45408-R	31 ± 8% of Standard TSH 2R3
317-51	280 ± 65% of Standard TSH 2R3
USP	300 ± 55% of Standard TSH 2R3

of assay, the doses 0.0125, 0.025, and 0.050 units of U.S.P. TSH have been used (these are approximately equivalent to 40 μg., 80 μg., and 160 μg. of thyrotropin sample 2R3). The 0.025 unit dose decreased the iodine contant of the chick thyroids 30–60% without usually affecting the thyroid weight.

DISCUSSION

Cockerels other than the White Leghorns used were tested for their sensitivity to TSH but were found to be unsuitable. The normal guinea pig and hypophysectomized rat were found to be very much inferior to the two day old White Leghorn cockerel. The guinea pig was found to be especially insensitive to TSH as 2.0 mg. of TSH #2R3 gave no decrease in thyroid iodine and only 35% increase in the mean thyroid weight. On the other hand, a dose of 2.0 mg. TSH #2R3 in the hypophysectomized rat caused a mean 18% decrease in thyroid iodine as compared to hypophysectomized controls and no increase in thyroid weight.

The White Leghorn cockereles have been found, thus far, to be the only breed of chicks very sensitive to thyrotropin. However, there were periods of variation in their sensitivity to TSH. These chicks seemed to be most sensitive in June and August, and least in April, November and December. To avoid variations in responses, a routine of injecting twice daily for three days was studied, but this resulted in responses no different than the one injection per day routine. This could be explained readily because of the prolonged action of TSH on the thyroid (see Table 2), the effect being

apparent 72 hours after a single injection of TSH. A one dose assay was then studied. One hundred chicks were injected once subcutaneously with 0.2 ml. solution, autopsied two days later, and the thyroid iodine determinations were performed as described above. The responses were poor. There was a 12% decrease in iodine at the 12 μg. level of 2R3, a 40% decrease at the 50 μg. level and only a 54% decrease at the 3.2 mg. level. Finally the thyroid weight response method was investigated and resulted in poorly graded, variable responses to graded TSH doses (0.4 gm. to 3.2 mg.). Consequently, the TSH preparations, in this laboratory, have been assayed using the iodine depletion of the thyroids of two day old White Leghorn cockerels as described above.

TABLE 2. THYROID IODINE OF CHICKS INJECTED ONCE WITH 3 MG. OF 2R3 AND AUTOPSIED AT VARIOUS INTERVALS

Hours after injection	Number of chicks	Average thyroid weight, mg.	Average* thyroid iodine, γ/mg.
0	10	3.6	2.26
0.5	10	3.4	2.39
1	10	3.8	2.25
2	10	3.5	1.96
3	10	4.7	1.71
4	10	4.3	1.65
7	10	4.3	1.59
24	10	3.4	1.46
32	10	3.3	1.41
48	10	3.4	1.27
72	10	4.2	1.31
72 (Control)	10	3.9	2.20

* One determination on the pool of all the thyroids of one group.

To note whether the thyrotropin potencies as obtained by this method were duplicable, two samples of TSH #317-51 and TSH #3 were subjected to several TSH assays over a period of several months using the chicks from one source (Berry's Hatchery, Illinois). These two TSH fractions were of different degrees of purity and activity; TSH #3 was a by-product of TSH fractionation while #317-51 was used in the preparation of the U.S.P. Thyrotropin Reference Substance. The results are shown in Table 3. The weighted mean standard errors obtained were 14.5% for TSH #3 and 7% for 317-51.

To note if the TSH contamination of other pituitary products could be detected by this method, a series of crude and potent hormonal fractions (other than TSH) were assayed. The results obtained (see Table 4) indicated that this assay method was very sensitive and could detect TSH contamination in anterior pituitary hormonal fractions at levels as low as 0.3% of 2R3.

TABLE 3. THYROTROPIN ASSAY RESULTS ON TSH #3 AND 317–51

Sample	Assay No.	Reference standard	Levels of sample per assay	TSH potency	
				% of 2R3	U.S.P. u./mg.*
TSH #3	160	2R3	1	170 ± 78	
	161	2R3	1	70 ± 34	
	162	2R3	1	93 ± 43	
	165	2R3	1	88 ± 20	
	166	2R3	1	103 ± 26	
Weighted Mean				89 ± 13	
317–51	149	2R3	2	270 ± 78	1.0 ± 0.3
	150	2R3	2	350 ± 140	1.3 ± 0.5
	153	2R3	1	180 ± 30	0.7 ± 0.1
	154	2R3	3	220 ± 28	0.8 ± 0.1
	165	2R3	1	280 ± 65	1.2 ± 0.3
	166	2R3	2	250 ± 43	1.0 ± 0.2
	167	2R3	1	340 ± 65	1.4 ± 0.3
	168	USP	1		0.6 ± 0.2
	171	USP	2		1.3 ± 0.4
	172	USP	2		0.9 ± 0.3
	173	USP	2		1.6 ± 0.2
	174	USP	2		1.4 ± 0.2
Weighted Mean				242 ± 17	1.1 ± 0.06

* 2R3 contains 0.37 U.S.P. units per milligram.

TABLE 4. TSH CONTENT OF SOME PITUITARY SUBSTANCES

Sample	% of standard TSH 2R3
Fresh beef anterior pituitary	0.8 ± 0.1
Beef anterior pituitary, acetone powder	5.0 ± 0.2
Sheep pituitary, acetone powder	1.7 ± 1.0
Fresh hog pituitary	1.0 ± 0.4
Hypothalamic extract	0
Prolactin, 2R-CR[1]	0
LH, FW-234[2]	9.7 ± 3.3
FSH, PFC-7C[3]	35.0 ± 11
Growth hormone 22KR2[4]	3.4 ± 3.1
ACTH 58-9(50)[5]	0.3 ± 0.1

[1] Contained 29 ± 8 International units of prolactin per mg.
[2] Assayed 113 ± 19 Collip units per mg.
[3] Assayed approximately 250 Collip units per mg.
[4] Armour's growth hormone standard having a potency of 100%.
[5] Assayed 1.8 International units ACTH per mg.

SUMMARY

A thyrotropin assay method based on the depletion of thyroid iodine has been developed which is dependent on a comparison (by means of the log dose method of Irwin) of responses obtained from unknown TSH preparations run at one or two levels to the responses obtained from two to three levels of a standard thyrotropin preparation. The two level assays gave TSH potencies with a standard error of approximately 25%.

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CORRELATION OF PREPARATIVE HISTORY AND METHOD OF ASSAY OF CORTICOTROPIN WITH CLINICAL POTENCY

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INTRODUCTION

IT IS well known that different ACTH assay methods give different results, but the reasons for this have not been fully elucidated.

Li (1952) has pointed out the discrepancies in evaluation of ACTH activity by different assay methods, which include the adrenal ascorbic acid depletion test of Sayers *et al.* (1948), the adrenal weight maintenance, and the hypertrophy and repair tests. Reinhardt *et al.* (1951) have emphasized the importance of applying several assay methods in the study of various ACTH preparations and the importance of absorption rates in assay methods. Discrepancies in potency estimates by the various assay means have also been noted by Young *et al.* (1951), Dixon *et al.* (1951) and Moyer *et al.* (1952). From the evidence presented there has frequently risen the postulate of two factors, one of which effects adrenal weight, and the second depletion of adrenal ascorbic acid.

Bruce, Parkes and Perry (1952) have studied the thymus involution response of nestling rats to ACTH and elaborated an assay method based upon this criterion of activity. Their method utilized litter-mate nestling rats, three daily injections of ACTH suspended in an oil-beeswax medium, and autopsy to obtain thymus weight twenty-four hours after the last injection.

In comparing the results of assay methods involving thymus or adrenal weight with the results obtained by the Sayers technique it must be borne in mind that the Sayers method utilizes intravenous injection of test doses while most other methods utilize subcutaneous or intramuscular injection.

There has been little clarification of the relative importance of routes of administration when different results have been obtained by different methods of assay. In addition, the variable results obtained by the several methods of assay have not been correlated with clinical activity. An abstract of the preliminary phase of these studies was reported by Fisher and Thompson (1952).

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MATERIALS AND METHODS

An experimental, purified ACTH in 16% gelatin standard was prepared for subcutaneous assay purposes. The term "purified" denotes preparations adsorbed and eluted from cellulose by methods originating in our laboratory and by the method described by Astwood *et al.* (1951). This standard was prepared by mixing equal unitage from each of three lots of purified ACTH in gelatin. These lots had been clinically assayed by several investigators and each was found to be two to four times as potent in terms of intravenously assayed International Units as other ACTH preparations in gelatin when administered subcutaneously or intramuscularly. On the basis of the assays on each lot by the intravenous method, and on the basis of intravenous assay of the finished mixture, the experimental standard contained 40 ± 4.0 I.U./cc. The standard has been stored frozen in small aliquots with no indication of potency change over a period of 12 months.

All test solutions contained the required dose of ACTH in 16% partially hydrolyzed U.S.P. gelatin (gel point about 23° C.) containing 0.5% phenol. All test samples in a given experiment were diluted with the same lot of gelatin.

The ACTH preparations used for all experiments reported (except when specified) in this study were derived from hog pituitary glands and were assayed in terms of I.U. to a standard error of 10% or less by the method of Munson *et al.* (1948) using hypophysectomized rats. All doses in subcutaneous experiments were given in terms of I.U.

The ACTH preparations referred to as crude have activities of less than 2 I.U./mg. as contrasted to the purified preparations, isolated by several different adsorption procedures, with activities between 20–60 I.U./mg. Preparations referred to as Type I forms are those which have been subjected to limited hydrolytic conditions during the process of isolation and the Type II and III forms are those subjected to more rigorous hydrolytic treatment, pepsin and pepsin plus acid, respectively. Types II and III are differentiated from Type IV in that the latter form has not been exposed to hydrolytic conditions.

The test animal in all cases was the Sprague-Dawley strain of rat. Intact and hypophysectomized animals of various ages were used as specified within each experimental design.

When adrenal ascorbic acid depletion was utilized as the end response following subcutaneous injection, individual groups of animals were sacrificed at suitable intervals after injection to obtain either a time response pattern or response at a specified time after administration of the hormone.

Preliminary experiments indicated that ACTH preparations prepared by different procedures were different in relative potency when assayed by the intravenous technique as compared to the subcutaneous technique employing either an aqueous or gelatin medium. Utilizing a gelatin medium, a greater response was obtained with relatively slight effect on the time pattern. A gelatin medium was employed throughout the following experiments because of the magnification of effect which could be obtained and because standardization of ACTH in gelatin was a primary problem.

All adrenal weight and thymus weight experiments reported in the present study involved the injection of 0.5 cc. test solution twice a day (9:00 A.M. and 4:00 P.M.) for three days, and autopsy the forenoon of the fourth day.

RESULTS

Figure 1 illustrates the results of a comparison of the adrenal ascorbic acid depleting activity of crude Type IV and crude Type I ACTH in saline, and in gelatin, after subcutaneous injection of 1.0 I.U./rat. Each point on the graph represents the average response of four rats. It may be seen that a gelatin medium produces a marked increase on the response

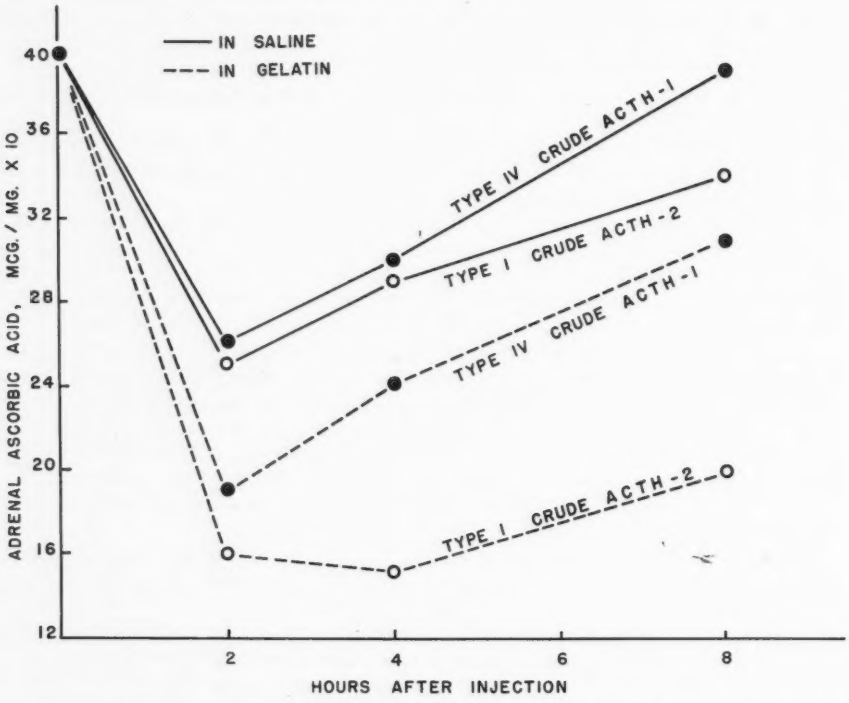

FIG. 1. Adrenal ascorbic acid response versus time after subcutaneous injection of two ACTH preparations in aqueous saline or gelatin vehicle. Each rat received 1.0 I.U. in 0.5 cc. Each point is the mean response value from four hypophysectomized rats.

to both types of preparations. However, the effect on the response to Type I ACTH appears considerably greater.

In order to determine the relative importance of "purity" as compared with "preparative history" in obtaining higher subcutaneous potency in a gelatin medium, the method was converted to a quantitative design and several suitable ACTH samples were assayed in comparison with each other. Figure 2 illustrates graphically the results of such an experiment. Rats were sacrificed at 2 and 4 hours after injection for determination of

adrenal ascorbic acid. Since there was no significant difference between the 2 and 4-hour response, these values were combined to produce the results illustrated in Figure 2. Each point then represents the average response of 8 rats.

In Figure 2, the ACTH sample identified as "Purified ACTH-247 (Type I)" assayed 30 I.U./mg., and the sample identified as "Crude ACTH (Type IV)" assayed 1.7 I.U./mg. When these samples in 16%

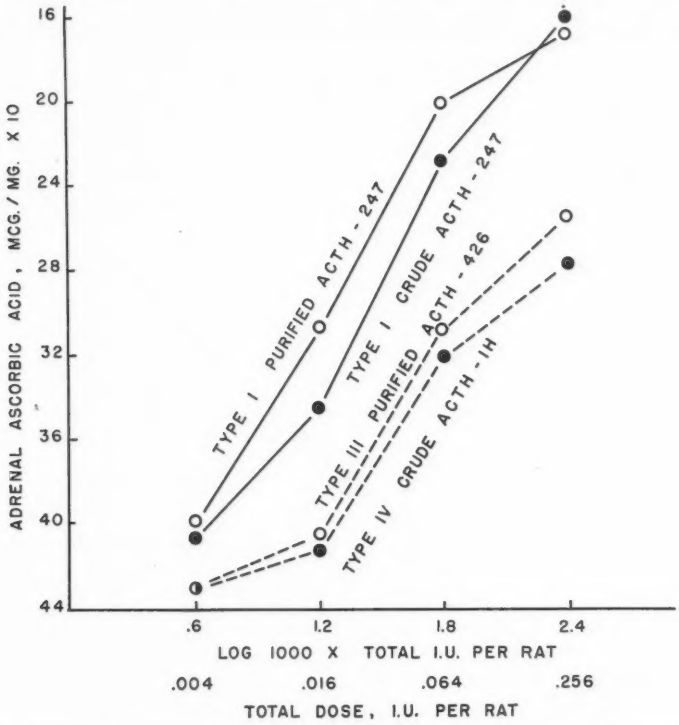

FIG. 2. Assay of several ACTH preparations subcutaneously administered and measuring adrenal ascorbic acid depletion in hypophysectomized rats—eight rats per point—three hour response.

gelatin medium were assayed clinically by intramuscular injection (Wolfson, 1952) the "Purified ACTH-247 (Type I)" was found to be two to four times as potent as the "Crude ACTH (Type IV)." It appears that there is good correlation between this assay method and clinical effectiveness. The sample identified as "Crude (Type I) ACTH-247" is the relatively crude extract (1.0 I.U./mg.) from which "Purified ACTH-247 (Type I)" was derived by selective adsorption. The sample "Purified

ACTH-426 (Type III)" isolated by chromatopile partition (White *et al.*, 1951) assayed 50 I.U./mg.

In view of the apparent correlation between clinical assays (subcutaneous or intramuscular administration) and the potency value obtained utilizing the adrenal ascorbic acid depletion method (subcutaneous administration), it was anticipated that other subcutaneous methods utilizing normal rats and/or other criteria of activity such as adrenal weight or thymus weight might also correlate with clinical activity.

An experiment on 23 day old normal Sprague-Dawley rats, selected at

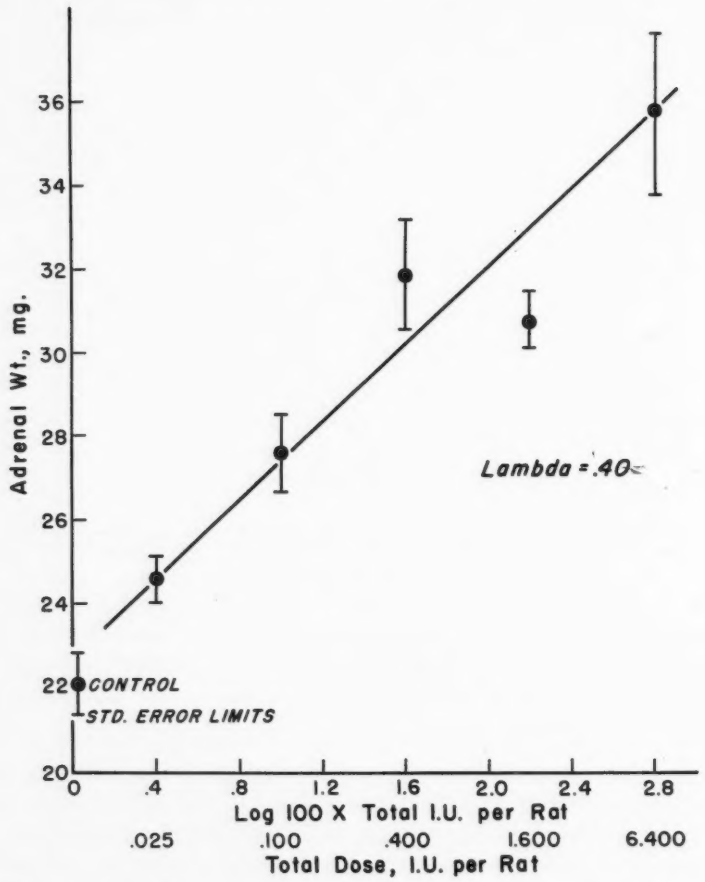

FIG. 3. Adrenal weight response of 23 day old, normal female rats to graded doses of purified ACTH in gelatin. 0.5 cc. subcutaneously twice a day for three days, autopsy fourth day, seven rats per point.

random, indicated that a useful adrenal weight response could be obtained on these animals but that the thymus involution response was more quantitative. Information from both responses could be obtained from the same rats (see also Hayashida and Li, 1952). One similar experiment on 30 day old rats indicated an adequate range of response using either adrenal weight or thymus weight but precision of the thymus response was poor in this experiment as compared to the 23 day old rats. Average control thymus weight was 323 mg. in this experiment and was reduced to 140 mg. with the highest dose of ACTH used (0.40 I.U. purified ACTH (Type I)—total dose in 3 days). Adrenal weight precision was comparable to results from 23 day old rats (lambda = .45).

Figure 3 illustrates the log-dose adrenal weight response relationship of purified ACTH (Type I) in gelatin administered as previously indicated to 23 day old normal female rats. The lambda value of 0.40 indicated relatively poor precision but is sufficiently valid to obtain information with

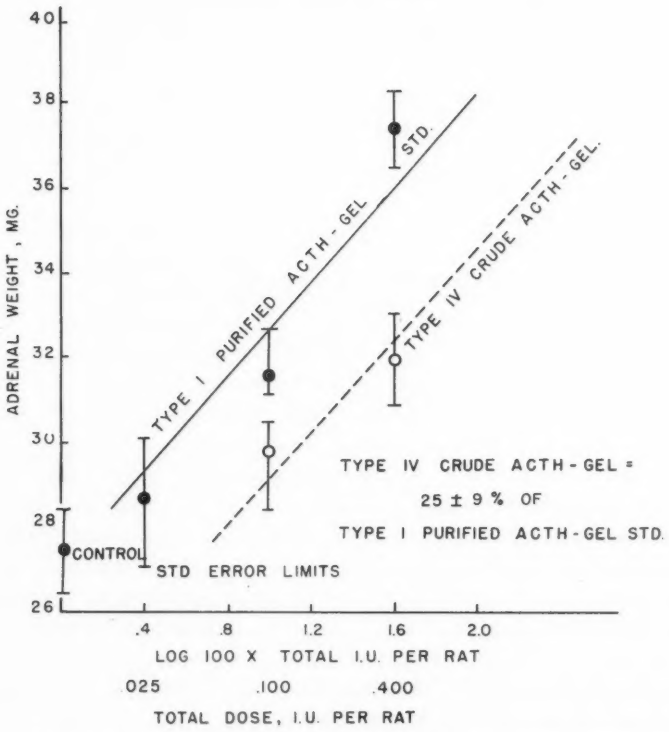

FIG. 4. Adrenal weight assay of ACTH. Thirty day old, normal, female rats—eight rats per point.

respect to correlation between adrenal weight assays and clinical or other assays.

Figure 4 illustrates the application of the adrenal weight response of normal 30 day old rats to the assay of crude ACTH (Type IV) in gelatin as compared to purified ACTH (Type I) in gelatin. The result—Type IV crude ACTH = 25 ± 9% of purified ACTH—is sufficiently accurate to indicate good correlation with clinical assay.

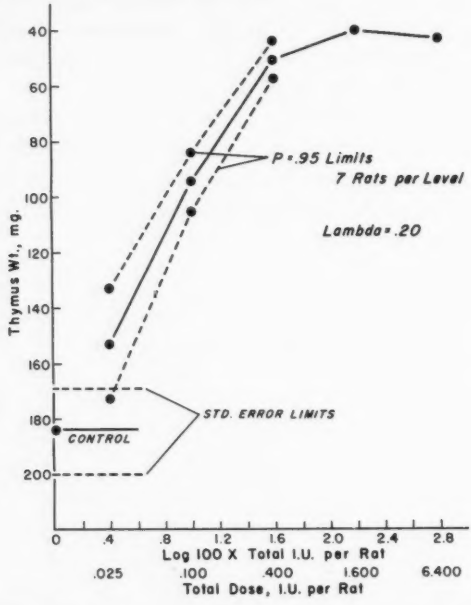

FIG. 5. Thymus weight response of 23 day old, normal, female rats to graded doses of purified ACTH in gelatin. This experiment also provided adrenal weight data shown in Figure 3.

Figure 5 illustrates the log-dose thymus weight relationship of purified ACTH (Type I) in gelatin administered to 23 day old female Sprague-Dawley rats selected at random. The thymus weights were obtained from the same rats that yielded the adrenal weight data illustrated in Figure 3. Thus it can be seen that in the dosage range of 0.10 to 0.40 I.U. of purified ACTH (Type I), quantitative data can be obtained simultaneously from both adrenal and thymus weight responses. In view of the correlation of standard deviation with level of response, conversion of the responses to log-response would seem indicated for assay purposes.

Figure 6 illustrates the assay curve obtained by using the logarithms of the thymus weight response values.

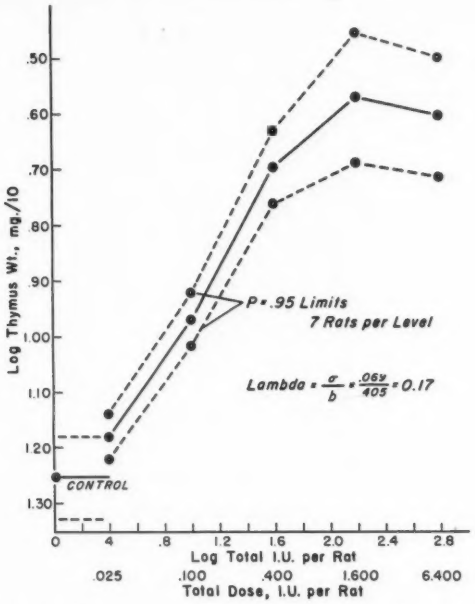

FIG. 6. Log thymus weight plotted against log dose of ACTH using data from experiment illustrated in Figure 5.

Figure 7 illustrates the application of the thymus involution assay procedure for the potency comparisons of several ACTH preparations. Again the comparison of crude ACTH (Type IV) in gelatin with the purified ACTH (Type I) in gelatin shows good correlation with clinical findings and with other subcutaneous assay methods.

In order to study further the application of several assay methods to the problem of ACTH standardization, additional assays were conducted on several samples of purified ACTH (Type I) in gelatin and crude ACTH (Type IV) in gelatin. All samples were first assayed by the provisional U.S.P. adrenal ascorbic acid depletion method on hypophysectomized rats to a standard error of $\pm 10\%$ (150 to 200 rats per sample). For subcutaneous assays, doses were always given in terms of I.U. or U.S.P. Units, so that all preparations can be considered to be 100% of each other, by the intravenous assay technique, within the limits of error indicated above.

Table 1 gives the results of the assay of several lots of purified ACTH (Type I) in gelatin and several lots of crude ACTH (Type IV) in gelatin when utilizing adrenal ascorbic acid depletion following subcutaneous administration in the hypophysectomized rat. It may be noted that each

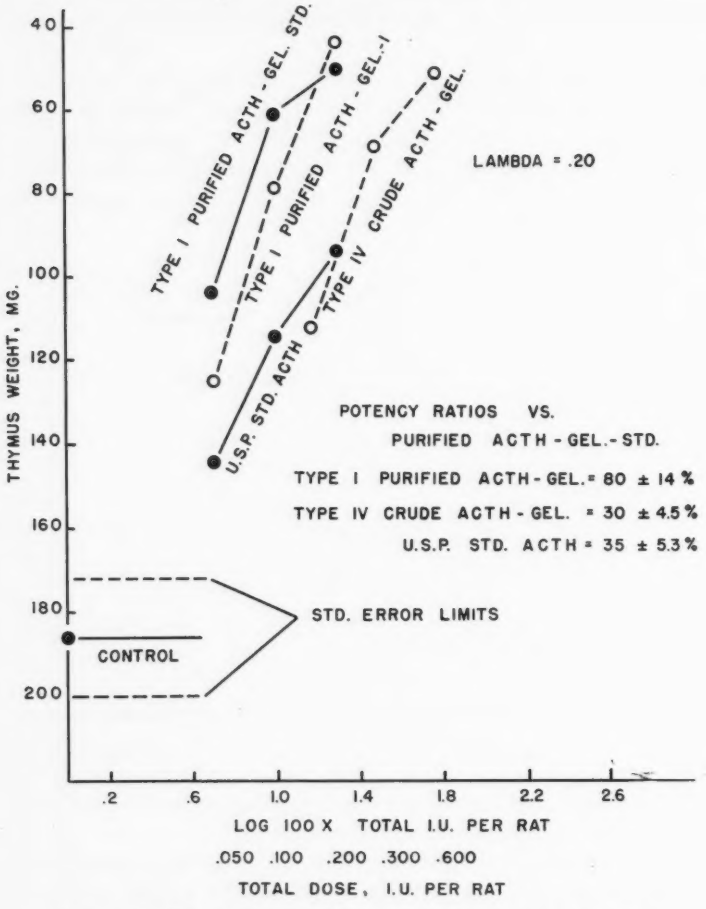

FIG. 7. Thymus involution assay of ACTH. Twenty-three day old, normal female rats. Seven rats per point.

type of preparation appears uniform within limits of error whether assayed intravenously or subcutaneously, but 1 I.U. of purified ACTH (Type I) in gelatin is about 2.9 times as potent subcutaneously (4-hour response) as is 1.0 I.U. of the crude ACTH (Type IV) in gelatin.

Different potency ratios between Type I and Type IV ACTH samples can be obtained by selecting different response times. Ratios at 2-hour response times may be about 2; at 4 hours, about 3; and at 8 hours, about 6–10. This is due to a difference in the time-response pattern (see Fig. 1). For standardization, samples should be assayed against a standard of similar type so far as preparative history and subcutaneous action characteristics are concerned.

TABLE 1. ASSAY OF SEVERAL ACTH PREPARATIONS, SUBCUTANEOUSLY ADMINISTERED, AND MEASURING ADRENAL ASCORBIC ACID DEPLETION IN THE HYPOPHYSECTOMIZED RAT

Purified ACTH type I in gelatin lot	Number of rats at each response time	Potency ratios versus purified ACTH experimental standard	
		2 Hours	4 Hours
1	30	.8	1.3
1	30	.6	.7
2	30	1.4	1.3
2	30	1.1	.9
3	30	1.4	1.2
4	24	1.3	1.7
5	20	.9	1.1
	Mean	1.07	1.17
Crude ACTH type IV in gelatin			
1	24	.47	.53
2	20	.53	.33
3	20	.47	.20
	Mean	.49	.35

1.0 I.U. purified Type I ACTH in gelatin = 1.0/.49 = 2.0 I.U. Type IV crude ACTH in gelatin by 2-hour response assay and 1.0/.35 = 2.9 by the 4-hour response assays.

The lambda values for the assays in Table 1 ranged from 0.2 to 0.25 so the individual assay results are subject to standard errors of about ±20 to 25% (20 to 30 rats per assay).

Table 2 gives the results of the assay of several lots of ACTH in gelatin preparations, by the thymus involution method utilizing 23 day old normal

TABLE 2. ASSAY OF SEVERAL ACTH PREPARATIONS, SUBCUTANEOUSLY ADMINISTERED, AND MEASURING THYMUS INVOLUTION IN 23 DAY OLD NORMAL RATS

Purified ACTH type I in gelatin lot	Number of rats	Potency ratios versus purified ACTH in gelatin experimental standard
1	42	.90
6	48	1.10
7	42	1.40
	Mean	1.13
Type IV crude ACTH in gelatin lot		
1—C	42	.30
1—C	48	.25
2—C	42	.32
U.S.P. standard in gelatin		
	42	.35
	Mean	.31

1.0 I.U. of purified Type I ACTH in gelatin = 1.0/.31 = 3.2 I.U. Type IV crude ACTH in gelatin.

female rats. The average lambda value in these experiments was 0.23 (0.20 to 0.31) so the individual assays are subject to a standard error of about ±16% and the means to a standard error of about ±8%. The purified ACTH (Type I) versus crude ACTH (Type IV) ratio of 3.2, therefore, exhibits close correlation with clinical results and with the other subcutaneous assay methods.

Table 3 gives the results of several assays on crude ACTH (Type IV) in gelatin assayed against the purified ACTH experimental standard by

TABLE 3. ASSAY OF SEVERAL ACTH PREPARATIONS, SUBCUTANEOUSLY ADMINISTERED, AND MEASURING ADRENAL WEIGHT INCREASE IN NORMAL RATS.

Type IV crude ACTH in gelatin lot	Number or rats	Potency ratio versus type I purified ACTH in gelatin experimental standard.
1	42	.28
1	48	.25
2	42	.37
U.S.P. standard in gelatin		
	42	.35
	Mean	.31

1.0 I.U. purified Type I ACTH in gelatin = 1.0/.31 = 3.2 I.U. crude Type IV ACTH in gelatin.

utilizing adrenal weight increase obtained in normal rats. Lambda values for these assays were 0.4 to 0.5 so the individual assays are subject to standard errors of about ±32% but the means are accurate to a standard error of about ±16%. By this method the relative subcutaneous versus intravenous potency of purified ACTH (Type I) in gelatin is about three times that of crude ACTH (Type IV) in gelatin.

DISCUSSION

The results indicate that if crude ACTH (Type IV) in gelatin were assayed by a subcutaneous method against the present U.S.P. standard, about the same value would be obtained as at present with the intravenous assay procedure. If the purified ACTH (Type I) in gelatin were assayed against the U.S.P. standard by the same method, a relative potency of about three times that of the intravenous assay technique would be obtained. This corresponds to the clinical assay of these two types of ACTH preparations. The inference is that, if preparations were assayed against the present U.S.P. standard by a subcutaneous method, the resulting products would be uniform in clinical activity when labeled in terms of U.S.P. units and used subcutaneously or intramuscularly. The clinical doses in terms of U.S.P. units would then be the same for either prepara-

tion. If such preparations were now labeled in terms of U.S.P. units as determined by the provisional U.S.P. intravenous assay procedure, one U.S.P. unit of one preparation would be about three times as potent clinically as one U.S.P. unit of another preparation, even though each preparation utilizes a gelatin vehicle. ACTH preparations in gelatin equivalent by the provisional U.S.P. assay are, therefore, not necessarily equivalent clinically following subcutaneous administration.

Some evidence exists (Fisher, 1953) that effective ranges of 0.5 to 3 times the U.S.P. unit may be encountered when comparing different type preparations. An experiment was designed to determine whether differences in dose response curve slopes or differences in time response pattern would be detectable after intravenous administration of Type I and Type IV ACTH preparations. The method used was the same as the Munson *et al.* (1948) assay procedure with the exception that rats were sacrifiecd at several time intervals. This experiment failed to show a difference between the two preparations comparable to that in the subcutaneous experiments. The time-response patterns appeared identical within limits of error. If a time difference in dose response curve slope exists, the purified Type I preparation shows the flatter slope. This latter feature is the opposite of findings after subcutaneous administration of the preparations. Comparison of the results of this experiment with the result of subcutaneous assays indicated that the major difference in relative potency of the two ACTH preparations appeared only after subcutaneous or intramuscular administration. The difference, therefore, must be due to a difference in rate of absorption or a difference in the rate of extravascular destruction.

The adrenal ascorbic acid depletion method, when ACTH is subcutaneously administered, discriminates quite sharply between preparations. The discrimination appears chiefly in the form of both deviation from parallelism of the log-dose response curve (Fig. 2), and differences in time-response pattern (Fig. 1).

All characteristics of the thymus involution experiment in Figure 5 indicate that this could be the basis for a simplified and more economical assay for ACTH than any previously available. Precision appears excellent in spite of the absence of litter-mate distribution. The method also retains the feature of discrimination between preparations in respect to their relative subcutaneous potencies. While only results obtained from the Sprague-Dawley strain of rats have been reported, other strains, Wistar for example, have proven satisfactory.

Response range is adequate. Even for randomly selected rats, precision (lambda = 0.23) appears as good as, or better than, other methods. The use of litter-mates may increase precision.

Since there appears to be a difference in the relative potency of different

ACTH preparations in different vehicles, it would be preferable to use the same vehicle for both standard and unknown.

For purposes of quantitative assay and standardization it is obvious that a new U.S.P. or International reference standard is needed. This suggested standard should be composed of material similar to the (Type I) purified preparations, both with respect to its purity and subcutaneous versus intravenous action characteristics.

When preparations have been assayed by all three of the methods described, the effect upon adrenal size or weight can be correlated to the effect upon adrenal ascorbic acid if the differences in intravenous and subcutaneous effectiveness have been initially evaluated. Preparations containing from 0.01 I.U. to 100 I.U./mg. as determined by intravenous assay methods have shown equal activity in respect to adrenal weight when administered subcutaneously in equivalent standard units. The foregoing studies appear to provide a sound basis for the use of subcutaneous assays of ACTH in normal animals.

The usual experimental design and methods of analysis are applicable. The best transformation of response in relation to linearity and standard deviation of response at different effect levels remains to be determined. Square root of response as suggested by Bruce *et al.* (1952) or log response as shown in Figure 7 should be satisfactory.

It is believed that the change in physiological effectiveness following acid and/or pepsin hydrolysis can possibly be related to the chemistry of the polypeptides isolated. The several facets of the aforementioned problem are under investigation at the present time.[1]

SUMMARY

ACTH has been prepared by different procedures and incorporated into a partially hydrolyzed gelatin vehicle. A gelatin medium enhances the effect of all preparations tested when given subcutaneously. The preparations were standardized by the provisional U.S.P. intravenous assay procedure using the adrenal ascorbic acid depletion in hypophysectomized rats. Some preparations were found to differ in potency by about 300% when injected subcutaneously in humans or in rat assay, even though initially standardized by the U.S.P. assay. Correlation between animal and human bio-assay of Corticotropin appears dependent upon the same mode of administration being used in both.

[1] Since receipt of this paper, the isolation of different purified ACTH preparations has been reported in the *J. Am. Chem. Soc.*, **75**, 245 (1953), as "Studies on Pituitary Adrenocorticotropin III. Differentiation of Three Active Types on XE-97 Resin" by W. F. White and W. Fierce.

Young *et al.* (1951), Dixon *et al.* (1951), and Moyer *et al.* (1952) have postulated the existence of multiple ACTH hormones as indicated by two different assay methods utilizing two different indices of response. Evidence is presented in this paper which indicates that by varying the route of administration (intravenous and subcutaneous) and utilizing the same response index (adrenal ascorbic acid), differences in relative potency appear. The magnitude of difference can be made parallel to that utilizing a second response index (adrenal weight) following subcutaneous administration. This fact would tend to contradict the postulate of two pituitary derived ACTH hormones, one affecting adrenal ascorbic acid depletion, the second adrenal weight.

ACTH preparations in gelatin, standarized by the provisional U.S.P. assay, were assayed by the adrenal ascorbic acid depletion method, an adrenal weight method, and a thymus involution method, all following subcutaneous administration of the hormone. All the above assays correlated satisfactorily with clinical findings. However, the thymus involution assay on 23 day old normal female rats appears to be the most promising.

Acknowledgments

The authors wish to extend their appreciation to Dr. E. E. Hays for his interest and cooperation during the course of these studies. They wish particularly to acknowledge the splendid cooperation, teamwork and technical skills of E. G. King, C. Johnson, W. Frey, W. Schauer and associates in conducting the various experiments involved.

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EXPERIMENTAL EXOPHTHALMOS IN RATS PRODUCED BY CORTISONE

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SOME time ago one of us (K. A.) noted that a rat with experimental fibrosis of the liver, for which it was being treated with cortisone, developed exophthalmos after it had received a total of 37.5 mg. of the hormone over a period of 5 days. Definite bilateral proptosis, confirmed by 3 independent observers, was present on the morning of the 6th day, but had decreased again on the 8th day despite the continued administration of cortisone. Close observation of other rats under similar treatment indicated the presence of varying degrees of proptosis, but owing to the difficulty of assessing the condition objectively, and owing to the additional complication of varying degrees of liver damage, it was decided to investigate the problem further in the guinea pig, which is a more suitable experimental animal for this purpose (Aterman, 1951, 1952a, b, c).

An opportunity to see if exophthalmos could be regularly produced in the rat presented itself again in the course of an experiment designed to study the effect of prolonged administration of cortisone on the growth of weanling male rats which, while on a fat-free diet, were receiving various dietary supplements e.g. fat, defatted liver, or desiccated thyroid. Details of the methods and of the diets used will be published elsewhere. Here it suffices to state that the onset and the course of the proptosis appeared to be independent of any dietary supplement and that, with a few exceptions, the only factor common to those animals in which proptosis occurred was cortisone.

METHOD

Of a total of 111 male Wistar rats weighing about 45–55 gm. at the start of the experiment 68 were given cortisone. The dose in each case was 1.5 mg. "Cortone" Merck,[1] diluted to 0.1 cc. with physiological saline, given subcutaneously daily for 6 days a week for 4 weeks. Each of the 68 rats therefore received a total of 36 mg. of cortisone acetate over a period of 28 days. The remaining animals were given daily injections of saline. The experiment was spread out over a period of months and ocular proptosis was observed in several groups of experimental animals.

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[1] We are indebted to Dr. Elmer Alpert of Merck & Co. for the cortisone acetate.

RESULTS

Fifty-seven of the 68 rats treated with cortisone looked, at some stage in the course of the experiment, as though they had protruded eyeballs. Figure 1 is a photograph of one such animal with its untreated control. Of the remaining 11 animals 5 died prematurely from intercurrent infections. In the control group of 43 rats injected with saline only 5 were considered to show some degree of ocular protrusion.

FIG. 1. Exophthalmos in a prepuberal male rat treated with cortisone. The control animal is on the left.

The rat is not a suitable animal for measuring the degree of exophthalmos by the usual caliper methods (Pochin, 1944). With experience it proved possible, however, to estimate the incidence as well as the degree of proptosis reasonably well by simple inspection. Table 1 shows the result of separate and independent attempts by 3 observers to grade the ocular changes in animals of which they only knew the numbers.

Unfortunately this method of assessment by 3 independent observers was not applied to all animals from the start of the experiment, so that definite statements about either the onset or the course of the proptosis in the cortisone treated rat cannot yet be made. In the majority of those animals which were watched from the beginning, proptosis became noticeable at some time between the 6th and the 13th day (6th day: 3 animals; 7th day: 6 animals; 8th day: 2 animals; 9th day: 7 animals; 13th day: 3 animals). In only one rat (No. 30) was exophthalmos thought to be present as early as the 3rd day after the start of treatment with cortisone. With the exception of this animal proptosis always appeared to be sym-

TABLE 1. AN ASSESSMENT BY 3 OBSERVERS OF THE DEGREE OF PROPTOSIS OF THE EYEBALLS IN A GROUP OF CORTISONE TREATED RATS AND THEIR CONTROLS

0 = No proptosis. x = Mild. xx = Moderate. xxx = Marked. xxxx = Gross.

Animal No.	Degree of proptosis present			Cortisone
	Observer			
	K.A.	S.M.G.	R.L.	
6	0	0	0	No
38	0	0	0	No
14	xx	xx	xx	Yes
7	0	x	0	No
13	xx	x	x	Yes
85	0	x	x	No
46	0	0	0	Yes
30	xxxx	xxxx	xxxx	Yes
47	x	x	0	Yes
55	0	0	0	No
23	0	0	0	No
29	xx	xxx	xxx	Yes
111	xxx	xxx	xx	Yes
112	x	0	0	Yes
77	0	0	0	No
51	0	0	0	No
104	xx	xx	xx	Yes
103	0	0	0	Yes
63	x	xx	x	Yes
22	0	0	0	No
39	xx	x	x	Yes
62	xx	xxx	xx	Yes
69	0	0	0	No

FIG. 2. Gross unilateral ocular protrusion of left eye with only mild proptosis of right eye in a male rat treated with cortisone.

metrical, and no other eye signs, such as the "bloody ring" described by Yudkin, Farquhar and Wakeman (1934) were observed. Proptosis was still present in many animals at the time of autopsy; in at least 4 animals, however, a previously established protrusion had disappeared.

Of particular interest was the course of exophthalmos in one animal, No. 30, to which reference has already been made. Proptosis was diagnosed in this animal by the 3rd day after the start of the cortisone injections. On the 11th day, nothing unusual was noticed, but on the 13th day, the left eye had literally left its socket (Fig. 2). Only moderate proptosis was present on the right side. The left eye as a whole appeared enlarged and close inspection revealed a certain degree of keratoconus. Of great interest was the occurrence here of the unilateral "bloody ring" noted by Yudkin *et al.* (1934) in some of their proptotic animals, a change which is presumably identical with the sanguineous discharge described by Thompson (1933). Pressure on the eyeball did not seem to cause any discomfort. The following day keratoconus was definite; the cornea had become completely dry and opaque; and the proptosis was unchanged. The picture was suggestive of a retroorbital tumour, and it was decided to kill the animal to avoid the possibility of a secondary infection preventing a correct diagnosis. The eye was punctured under light ether anaesthesia and clear fluid escaped under pressure, presumably from the anterior chamber. This undoubtedly was the cause of the keratoconus and the apparent enlargement of the eye, but it did not account for the proptosis, which still persisted. Enucleation showed that the eyeball was not appreciably enlarged. The retroorbital contents appeared hyperaemic, but no tumour or pus could be found. This was confirmed by histological examination (Fig. 3).

This peculiar picture was not observed in any other rat in this or any other series of similar experiments. It is possible that it is similar to the rare unilateral exophthalmos of endocrine origin in man.

DISCUSSION

Yudkin *et al.* (1934) observed proptosis in young male albino rats that were being given diets low in salts. Of 34 rats 11 developed marked and 11 slight and occasionally transient ocular protrusion. A "bloody ring around one eye" was noted in 4 animals. No explanation for this unilateral occurrence is given. On histological examination no evidence of increased ocular tension could be found, nor were there any changes present in either the eye muscles or the retrobulbar tissues. The thyroid is said to have shown "striking changes." Loss of hair was recorded in some animals, but no abnormality was reported in the pituitary or the adrenals, the weights of which, however, are not given.

Somewhat similar findings have been reported by Thompson (1933),

who kept rats on diets with varying calcium and iodine contents. Ocular disturbances occurred in the form of proptosis and decreased frequency of blinking. Occasionally a sanguineous exudate developed around the lids of one or both eyes. Proptosis became noticeable about the sixth week; reached its maximum during the eighth to tenth week; and remained unaltered from then on. These eye changes were limited to animals on diets containing little iodine, in whom hyperplasia of the thyroid glands,

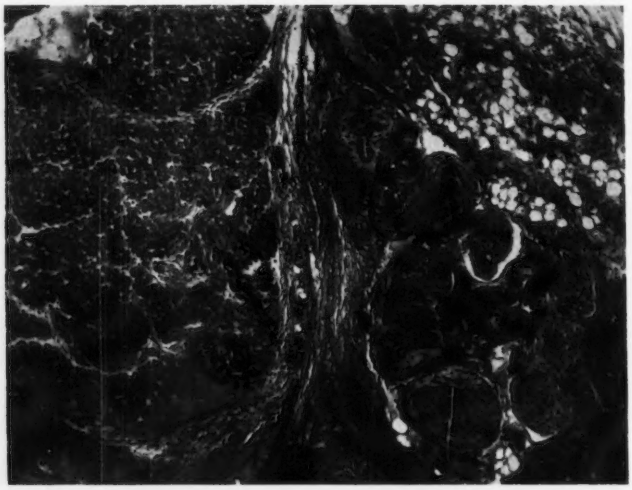

FIG. 3. Section of the left orbital tissues of the rat with asymmetrical proptosis. There is no evidence of inflammation or tumour present. (Formalin-Zenker, Hematoxylin-Eosin, 7μ×75).

followed later on in some cases by atrophy, was found. Apart from changes in the parathyroid glands the state of other endocrine organs is not mentioned.

Heinemann (1937) produced exophthalmos in guinea pigs and rats by injecting thyrotropic hormone (Schering-Kahlbaum). At autopsy the thyroids were shown to be enlarged. Exophthalmos occurred in animals treated for more than 2 weeks, and was most marked in those animals which had been treated longest. There was no loss of weight, but loss of fur was noted. The adrenal glands in the exophthalmic guinea pigs were definitely enlarged.

Definite exophthalmos persisting after death has been reported by Rawson, quoted by Means (1944), in rats in which a hyperplastic goitre was produced by treatment with sulfathiazole. Sellers and Ferguson (1949) have also observed proptosis in rats fed with propylthiouracil for

prolonged periods. It could be seen in some animals after only 3 months, but it took 7 months before it was present in almost every rat. It was best observed if the animal was active and alert. All rats were under weight and had scanty, coarse hair. The weight of the eyeballs was not larger than in the control group, and no lymphocytic infiltration was present in the retrobulbar tissues.

It is generally stated that exophthalmos is due to excessive production of the thyrotropic hormone. The occurrence of thyroid changes in the experiments just quoted could be interpreted as supporting this thesis. Since the prolonged administration of cortisone or ACTH is said to induce thyroid depression—the "corticogenic hypothyroidism" of Wolfson *et al.* (1950, 1951)—the occurrence of proptosis in cortisone-treated animals could be explained on the basis of a subsequent increase in the secretion of the thyrotropic hormone. Some clinical (Richardson *et al.* 1950; Reiss *et al.* 1950; Werner, 1950); and experimental (Winter *et al.*, 1950) findings do not, however, support this view. Since it has furthermore been shown that cortisone significantly increases the thyrotropin-induced exophthalmos in normal as well as in thyroidectomised guinea pigs (Aterman, 1952b, c), it is difficult to maintain the thesis that this cortisone effect is mediated through the thyroid. Even if cortisone can affect the thyroid as an indirect consequence of some change in the pituitary, it is also probable that the hormone acts directly on the connective tissues of the body. The explanation of the ocular protrusion observed in the present experiments would then be the changed physical state of these tissues within the orbit (Aterman 1952b, c).

Reference has already been made to the unusual course of exophthalmos in rat No. 30 (Fig. 2). No explanation can be advanced for the exceptional features present in this animal. Since an increase, no change, or a decrease in the intra-ocular tension under the influence of ACTH or cortisone have been reported under various clinical and experimental conditions (Bardram, 1950; Fitzgerald *et al.* 1951; Olson *et al.* 1951; Sallman *et al.* 1952; Stewart Duke-Elder, 1951), it is questionable whether the increased tension present in the proptosed left eye of this rat can be attributed to a direct effect of cortisone or whether it was the result of the pronounced exophthalmos. The marked asymmetry of the latter also remains unaccounted for. Although some of the rats in the present series developed quite a marked degree of exophthalmos, we have not been able to reproduce a similar picture. Unilateral exophthalmos in experimental animals has, however, been reported by other workers (Friedgood, 1934; Smelser, 1937; Thompson, 1933). The absence of any macroscopic or microscopic evidence of a retroorbital tumor or inflammation (Fig. 3) in our experimental animal suggests a similarity with the rare but well known cases of

"unilateral" endocrine exophthalmos in man occasionally mistakenly diagnosed as retroorbital tumors and even operated on (Anderson, 1947; Brain, 1936, Bedrossian, 1951; Burch, 1929, 1942; Denis and Mahaux, 1951; Drescher and Benedict, 1950; Jensen, 1940; Kisner and Mahorner, 1947; Klotz, 1948; Regnier, 1942; Rundle and Wilson, 1944, 1945; Saltzmann and Mellicker, 1951). That endocrine factors should lead to asymmetrical changes in symmetrical organs is a puzzling, but not unknown phenomenon of which several instances can be found in the literature (Bond, 1914; Greene, 1948; Peyton, 1942; Wilkins, 1948).

SUMMARY

The occurrence of varying degrees of bilateral proptosis has been observed in young male Wistar rats treated for 4 weeks with cortisone acetate. In one animal marked asymmetry of the proptosis occurred. These findings are discussed in the light of our present knowledge of the etiology of exophthalmos.

Acknowledgment

We should like to thank Miss R. H. Lawrence for her valuable assistance.

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NITROGEN BALANCE, LIVER PROTEIN REPLETION AND BODY COMPOSITION OF CORTISONE TREATED RATS

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IN 1940, Long, Katzin and Fry demonstrated that the administration of adrenal cortical extract to fasting rats caused a loss of nitrogen in the urine and an increase in liver glycogen. They suggested a relationship between protein breakdown and glycogen synthesis in such animals. For reviews of subsequent investigations, the reader is referred to those of Ingle (1950), Sayers (1950) and Engel (1951).

When cortisone acetate[1] became available in 1949, it seemed desirable to investigate further the effects of cortisone on nitrogen balance, on liver protein regeneration, and on the distribution of protein and fat in the body.

EXPERIMENTS AND RESULTS

Male albino rats of the Carworth strain were selected from single shipments and grouped to obtain equivalent average body weights. Adrenalectomies were performed by the lumbar approach under nembutal anesthesia and operated rats were given 1% saline to drink. All animals were housed in individual mesh-bottom cages in air conditioned quarters during the experiments. Nitrogen was determined by the micro-Kjeldahl method. References to other analytical techniques accompany the data.

The probability (p) of erroneously concluding that there are significant differences between means was calculated by Student's t test or from the variance ratio (F). See Snedecor (1946).

a) *Nitrogen balance.* Rats weighing 250 gm., on the average, were fed protein-free diet (Silber and Porter, 1950), *ad libitum* for 7 days. On the 8th, 9th, and 10th days, consumption of the diet was limited to 8 gm./rat /day and each of thirty-two rats was given daily subcutaneous injections of 3 mg. cortisone acetate in aqueous suspension. A second group of thirty-two rats did not receive cortisone. On the 9th day, all rats were placed in metabolism cages in groups of two. On this and the subsequent day, half of the cortisone-treated rats and half of the untreated rats each received, in addition to the daily allotment of protein-free diet, an oral dose of a solu-

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[1] Cortisone Acetate (Merck).

tion of amino acids or egg albumin (300 mg. N/kg. rat). Twenty-four hours after the second dose, the rats were given 10 ml. of water orally, and urines were collected with cage washings two hours later. The urinary nitrogen data are summarized in Table 1.

TABLE 1. THE EFFECT OF CORTISONE ACETATE UPON NITROGEN BALANCE. NITROGEN GIVEN, 300 MG./KG./DAY

Group	Treatment		Av. rat wts. before test, gm.	3-day wt. loss, gm.	Liver wt., % body wt.	Av. urinary N, mg./kg./day ±s.e.
	Cortisone	Amino acids				
			Amino acid experiment			
1–4	+	+	226	11.5	4.1	474 ± 7.2
5–8	+	0	225.5	15.5	3.7	365 ± 24.1
9–12	0	+	227	2.0	3.2	372 ± 5.4
13–16	0	0	226	8.0	3.0	267 ± 9.7
			Egg albumin experiment			
	Cortisone	Egg albumin				
1–4	+	+	243	—	—	367 ± 7.2
5–8	+	0	239	—	—	338 ± 25.0
9–12	0	+	239	—	—	272 ± 17.3
13–16	0	0	238	—	—	231 ± 6.4

Sixty-four per cent of the orally administered amino acid nitrogen was retained by the cortisone-treated rats and 65% by the controls. The retention of egg albumin nitrogen also was comparable in the two groups, 90% and 86%, respectively. Expressed as nitrogen balance indices (Allison, Seeley, and Ferguson, 1947) these values would be 0.64, 0.65, 0.90, and 0.86. Thus, cortisone had no effect on the retention of ingested nitrogen. The rats lost 13 gm. body weight/kg. and 100 mg. urinary nitrogen/kg./day more than their controls. Nevertheless, the livers of the rats given cortisone contained 200 mg. more nitrogen/kg. body weight than those of the controls.

b) *Liver protein repletion.* Rats which were adrenalectomized on the fourth day of protein depletion were supplied protein-free diet for six additional days. On the 11th, 12th, and 13th days some were allowed to consume 4.5 gm. protein-free diet, others 4.5 gm. 20% casein diet per 100 gm. body weight each day. Cortisone injections (1.5 mg./100 gm. body wt.) were made daily from the fourth to the thirteenth day, inclusive. Intact rats were treated similarly.

No significant difference was observed between the intact and the operated rats so the data were pooled, as shown in Table 2. The livers of the repleted, cortisone-treated rats were larger and contained more protein nitrogen, more NPN and more glycogen. Serum protein concentrations

TABLE 2. THE EFFECTS OF CORTISONE ON LIVERS OF RATS DURING PROTEIN-REPLETION

	Cortisone-treated (13 rats)	Controls (9 rats)
Liver wt., gm./100 gm. rat	5.28 ±0.16	3.18 ±0.061
Protein N. mg./100 gm. rat	127.8 ±1.7	94.6 ±2.0
N. P. N., mg./100 gm. rat	17.9 ±0.35	13.0 ±0.27
Glycogen, mg./100 gm. rat	429.8 ±36	48.8 ±5.9

were also increased (6.6 versus 5.7 gm.%). Similar but less striking differences were seen in the rats which remained on protein-free diet throughout the experiment.

The subsequent experiment was designed to study the quantitative relationship between the effects of cortisone and protein feeding upon liver protein regeneration. Adrenalectomized rats were fed protein-free diet *ad libitum* from the fourth through the seventh day after operation. They were then allowed to consume 5 gm. per 100 gm. body weight per day of protein-free or 15% casein diet, and some were given daily subcutaneous injections of cortisone acetate (2 mg./100 gm. rat). Rats were sacrificed two or four days after the first injection. Analysis of variance of the data (Table 3) shows that the very significant effects of cortisone and of dietary protein upon liver nitrogen had no influence upon one another.

c) *Effects of cortisone on carcass and viscera of rats.* From the above results, it was clear that cortisone could cause an increase in liver and serum protein while nitrogen was being lost in the urine, whether or not the animals were supplied dietary protein. The following experiment was designed to locate the source of the endogenous nitrogen available for liver regeneration and for excretion.

Five days after adrenalectomy, groups of five rats were injected daily with 0.1 or 1.0 mg. cortisone acetate per 100 gm. rat, and allowed to consume 3.5 gm. of protein-free diet per 100 gm. rat/day. Control animals were not included because adrenalectomized rats do not survive on the protein-free diet in spite of saline therapy.

The rats were sacrificed after 14 days. They were exsanguinated, and the carcasses were divided as follows: (1) liver, (2) spleen, (3) gastro-intestinal organs (washed stomach and intestines, and adhering structures), (4) urogenital organs (testes, prostate, kidneys, bladder), (5) pleural organs (heart and lungs), (6) carcass (muscle and bone, including the head and brain), and (7) skin and hair.

Livers and spleens were homogenized with water and aliquots taken for the various analyses. The other tissues were macerated in a Waring Blender with 70% alcohol, then extracted in Soxhlet extractors with 95% ethanol, then ether. The extracts were evaporated to dryness and the residues

TABLE 3. EFFECT OF PROTEIN AND CORTISONE UPON LIVER N REGENERATION IN ADRENALECTOMIZED RATS

5 rats/group; saline *ad libitum*

Protein	Cortisone	Days	Body wt. at start, gm.	Body wt. at sac., gm.	Liver N, mg./100 gm. rat ± s.e.
0	0	2*	190	178	85.9 ± 2.76
0	0	4*	190	168	84.3 ± 1.40
0	+	2	190	181	101.8 ± 1.18
0	+	4	190	172	110.1 ± 3.39
+	0	2	190	189	99.0 ± 2.84
+	0	4	190	193	102.4 ± 2.06
+	+	2	190	183	126.4 ± 1.68
+	+	4	190	186	125.3 ± 3.50
		0	190	190	92.2 ± 2.40

* These group consumed 3.5 and 2.5 gm. diet/100 gm. rat/day. The remaining groups consume l all 5 gm. of their diets/100 gm. rat/day.

ANALYSIS OF VARIANTS

Source of variation	Degrees of freedom	Mean squares	F*	p
Protein	1	3766.57	100.79	<0.005
Cortisone	1	6352.60	169.99	<0.005
Days	1	59.86	1.60	
Protein × Cortisone	1	56.77	1.52	
Protein × Days	1	14.95	0.40	
Cortisone × Days	1	21.60	0.58	
Protein × Cortisone × Days	1	156.24	4.18	0.05
Error	40	37.37		

* F for 1 and 40 d.f., (p = 0.005) = 8.83; (p = 0.05) = 4.08.

partitioned between water and petroleum ether. The defatted tissues were dried and pulverized, and aliquots were taken for analysis, except in the case of skin, where the whole sample was used. For protein analyses samples of dried defatted carcasses and the skins were extracted with N/10 NaOH. Three 24-hour extractions at room temperature were sufficient to remove essentially all of the soluble proteins from the carcass samples. The skins were extracted four times, with freezing and thawing, to remove the soluble proteins. Collagen in carcasses and skins was determined essentially according to Lowry, Gilligan and Katersky (1941). Total nitrogen on the residues from the collagen extractions of the carcasses is reported here as "elastin" N; the figure also includes bone nitrogen. Since the hair was not separated from the skins, total nitrogen on skin residues was not determined.

The rats which received the higher dose of cortisone lost significantly more weight than those receiving the lower dose (Table 4). The carcasses and skins lost weight roughly in proportion to body weight. The internal organs maintained or increased in weight, so the organ weight to body weight ratios showed significant increases. The analytical data on blood

TABLE 4. ORGAN WEIGHTS, SERUM PROTEIN AND LIVER ANALYSES. ADRENALECTOMIZED PROTEIN-DEPLETED RATS TREATED WITH 0.1 OR 1.0 MG. CORTISONE ACETATE/100 GM. RAT/DAY

	Low dose	High dose	p
Initial body wt., gm.	229	229	
Body wt. loss, %	27.3 ± 0.96	30.8 ± 0.33	<0.025
Carcass wet wt., gm./100 gm. rat	60.2 ± 0.83	58.0 ± 0.59	<0.1
Dry, defatted, carc. wt., gm./100 gm. rat	16.8 ± 0.20	16.4 ± 0.38	>0.2
Skin and hair wet wt., gm./100 gm. rat	18.1 ± 0.38	17.3 ± 0.21	<0.2
Dry, defatted skin wt., gm./100 gm. rat	6.08 ± 0.18	6.06 ± 0.08	>0.2
Liver wt., gm./100 gm. rat	2.93 ± 0.07	4.18 ± 0.12	<0.005
Liver dry wt., gm./100 gm. rat	0.75 ± 0.022	1.14 ± 0.042	<0.005
Viscera wt., gm./100 gm. rat	11.3 ± 0.29	12.7 ± 0.51	<0.05
Dry, defatted viscera wt., gm./100 gm. rat	1.31 ± 0.024	1.46 ± 0.055	<0.05
Total serum protein, gm. %*	5.27 ± 0.16	6.00 ± 0.071	<0.005
Albumin (pooled sample), gm. %	1.45	2.12	
Globulin (pooled sample), gm. %	3.72	3.93	
A/G ratio	0.39	0.54	
Liver protein N, mg./100 gm. rat	76.5 ± 1.31	105.5 ± 1.86	<0.005
Liver NPN, mg./100 gm. rat	9.47 ± 0.30	13.51 ± 0.34	<0.005
Liver carbohydrate, mg./100 gm. rat	137 ±23.4	320 ±20.5	<0.005
Liver lipid (alc.-ether sol.) mg./100 gm. rat	261 ±22.3	452 ±24.6	<0.005
Liver desoxyribonucleic acid, mg./100 gm. rat†	15.2 ± 0.39	17.1 ± 1.26	<0.2
Liver ribonucleic acid, mg./100 gm. rat‡	43.8 ± 3.56	68.4 ± 4.10	<0.005

* Total protein and protein fractions by the method of Kibrick and Blonstein (1948).
† Dische (1930).
‡ Davidson and Waymouth (1944).

serum showed that cortisone prevented a decrease or caused an increase in albumin, even though these animals were consuming protein-free diet, but there was little change in globulin.

As the livers increased in size under the influence of the higher dose of cortisone, protein and fat (alcohol-ether soluble) increased in about equal proportions while carbohydrate increased to a greater extent. Liver ribonucleic acid increased in proportion to liver weight, but desoxyribonucleic acid remained essentially constant in quantity, indicating an increase in cytoplasm without an increase in the number of cells. (The relative increase in desoxyribonucleic acid indicated in the table was of doubtful significance, $p < 0.2$, and was at least partially a result of the decrease in body weight.)

The distribution of nitrogen in the animals (Table 5) closely paralleled the organ and body weight distribution discussed above (Table 4). Total nitrogen of the rats decreased proportionately with body weight loss. However, there was an excessive loss of nitrogen from the carcass whereas the liver, gastro-intestinal organs and urogenital organs were spared nitrogen loss. The collagen and elastin nitrogen of the carcass showed a relative and absolute increase, while the more labile alkali-soluble nitrogen fraction decreased significantly. The fraction of nitrogen contributed by the skin remained the same in the two groups.

The ratio of total fat (Table 6) to body weight increased by 50%

TABLE 5. NITROGEN DISTRIBUTION IN ADRENALECTOMIZED, PROTEIN-DEPLETED RATS TREATED WITH 0.1 OR 1.0 MG. CORTISONE ACETATE/100 GM. RAT/DAY

	Low dose	High dose	p
Nitrogen, % of rat total N (skin elastin and hair omitted)			
Carcass	68.0 ±0.49	65.0 ±0.43	<0.01
Skin	22.1 ±0.45	22.6 ±0.32	>0.2
Liver	3.03±0.085	4.49±0.114	<0.005
Spleen	0.30±0.055	0.25±0.019	>0.2
Pleura	0.25±0.004	0.25±0.007	>0.2
Gastro-intestinal	3.66±0.070	4.27±0.130	<0.01
Urogenital	2.55±0.073	2.96±0.120	<0.05
Total N gm./100 gm. rat	2.79±0.061	2.67±0.037	>0.2
N of carcass fractions, % of carcass total N			
Water solubles from Soxhlet extract	5.84±0.28	6.77±0.27	<0.1
Alkali soluble	82.30±0.57	76.87±0.48	<0.01
Collagen	10.16±0.54	14.24±1.00	<0.025
Elastin & bone	1.67±0.088	2.11±0.12	<0.05
Carcass total N, gm./100 gm. rat	1.90±0.053	1.74±0.031	<0.1
N of skin fractions, % of skin total N (hair & elastin omitted)			
Water soluble from Soxhlet extract	6.84±0.34	5.84±0.33	<0.2
Alkali soluble	41.56±0.53	41.16±1.12	>0.2
Collagen	51.48±0.88	52.90±1.17	>0.2
Soluble N+Collagen N, gm./100 gm. rat	0.62±0.017	0.61±0.001	>0.2

($p < 0.1$) under the influence of cortisone. Particularly striking increases in fat were observed in the carcass and organs of the pleural cavity and less striking increases in the urogenital organs and livers. Fat in the skin and gastro-intestinal organs remained essentially constant.

DISCUSSION

Since the report of Long, Katzin and Fry (1940) cortisone has frequently been termed a "catabolic" hormone. From isotope experiments, Hoberman (1950) and Clark (1950) have considered cortisone to exert catabolic or

TABLE 6. FAT* IN ADRENALECTOMIZED PROTEIN-DEPLETED RATS. TREATED WITH 0.1 OR 1.0 MG. CORTISONE ACETATE/100 GM. RAT/DAY

	Low dose	High dose	*p*
Carcass, gm. fat/100 gm. rat	1.46±0.14	2.62±0.35	<0.025
Skin, gm. fat/100 gm. rat	0.91±0.22	1.03±0.09	>0.2
Liver, mg. fat/100 gm. rat	115±11.4	158±20.8	<0.2
Pleura, gm. fat/100 gm. rat	41.5±5.9	95.3±11.8	<0.005
Gastro-intestinal, mg. fat/100 gm. rat	317±159	588±137	>0.2
Urogenital, mg. fat/100 g. rat	451±101	731±81	<0.1
Spleen, mg. fat/100 gm. rat	14.2±2.5	16.3±1.3	>0.2
Total fat, gm./100 gm. rat	3.30±0.62	5.24±0.63	<0.1

* Benzin soluble.

anti-anabolic effects on protein metabolism. Ingle (1950) and Engel (1951) have suggested that the primary effect of cortisone may be a decrease in the utilization of carbohydrate. The fact that cortisone in milligram quantities can depress the body weight of rats without a proportionate decrease in food intake is well substantiated. From the observations of Kochakian and Robertson (1951) and from the present work, it is clear that the loss of body weight is due to loss of substance, including nitrogen, from the carcass rather than from the internal organs.

Loss of nitrogen from the carcass was not accompanied by deficient utilization of ingested protein as evidenced by nitrogen balance and liver protein repletion. This is in agreement with the report of Wells and Kendall (1940) that adrenalectomy did not influence the utilization of exogenous protein.

Engel, Schiller, and Pentz (1949) found that in nephrectomized rats, exogenous amino acids or glucose spared the breakdown of endogenous protein caused by injection of adrenal cortical extract. In the present experiments, however, the effect of cortisone upon the incorporation of nitrogen in the liver or its excretion in the urine was not influenced by the ingestion of minimal amounts of protein by intact rats. In rats fed normal amounts of dietary protein Clark (in press) found that cortisone treatment caused an increase in the excretion of isotopic nitrogen fed as glycine. The apparent contradiction between this and the present work may be related to the level of nitrogen intake.

In the nitrogen balance experiment it was noted that the body weights of the rats given cortisone decreased 13 gm./kg./day more than their controls in spite of equivalent caloric intakes. If the tissue protein is assumed to contain 16% nitrogen and the wet tissue to contain 20% protein, only one-fifth of the weight loss can be related to the loss of nitrogen in the urine. There was no evidence in these experiments that dehydration occurred in the carcasses of the cortisone-treated rats although they did excrete larger volumes of urine. They also consumed more water, and dry weight data showed that the water concentrations in the carcasses were comparable in the treated and untreated groups.

The liver protein of cortisone-treated rats decreased in concentration, but increased in total, This may be related to the deposition of glycogen and the increase in cell size. However, the elevated serum albumin concentrations found in the cortisone-treated rats also indicates an increase in protein synthesis by the liver. Mushett, Porter and Silber (unpublished) have found that cortisone-treated dogs on stock diet *ad libitum* showed changes similar to those observed in rats, i.e., in liver protein, fat, and nucleic acid. Friedgood, Vars and Zerbe (1950) have reported that cortisone facilitated liver regeneration after partial hepatectomy. Kochakian and

Robertson (1951) called attention to the fact that protein anabolism may occur in certain tissues simultaneously with catabolism in others. It seems reasonable to conclude that the protein catabolic (or anti-anabolic) effects of cortisone were, under the conditions of our experiments, essentially limited to the labile carcass proteins.

SUMMARY

In nitrogen balance and liver protein repletion experiments cortisone did not alter the biological value of an amino acid mixture, egg albumin or casein.

In protein-depleted rats, cortisone caused a wastage of nitrogen from carcass fractions other than structural protein (collagen and elastin). Concurrently, the total protein of the liver, gastro-intestinal tract and urogenital organs, as well as plasma albumin were increased. The increased size of livers caused by cortisone may be due primarily to a laying down of cytoplasm (ribonucleic acid increased) without the formation of new cells (desoxyribonucleic acid not increased). An increase in total fat of the animal was observed, with the most significant increases occurring in the carcass and organs of the pleural cavity.

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RADIOIODINE METABOLISM IN THE CHICK EMBRYO

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INTRODUCTION

MANY studies have been made of the histology of the thyroid gland at various stages in the development of the chick embryo. These studies, as well as several to determine the age of the chick embryo at which biochemical differentiation or thyroid function begins, have recently been summarized by Moore (1950). Methods used to determine thyroid function in the chick embryo include measurement of the total iodine in the thyroid glands (Sun, 1932), demonstration of the presence of the thyroxine in the chick thyroid by tadpole test (Hopkins, 1935), and demonstration of organic binding of radioiodine in the thyroid gland by autoradiography (Hansborough and Khan, 1951). Using these methods, it was not possible to detect any evidences of thyroid function or biochemical differention prior to the tenth day of incubation, which is the time colloid has been reported to appear in the thyroid gland (Bradway, 1929; Hopkins, 1935; Venzke, 1949).

It has been recognized that in addition to its ability to incorporate iodide into organic binding and to synthesize thyroxine, the thyroid gland differs from non-thyroid tissue in that it has the property of maintaining a concentration of iodide greatly elevated above that of the blood (Albert, 1952).

The investigation reported in this paper was undertaken to determine the temporal relationships involved in the development of the ability of the embryonic thyroid gland to maintain a concentration of iodide above that of blood and the ability to incorporate iodide into organic binding. Radioiodide and quantitative counting procedures were used.

It was found that the ability of the thyroid gland to concentrate iodide from the blood develops before its ability to incorporate iodide into organic binding. Iodide concentrating occurred before follicle formation at a stage when there was not more than a trace of colloid in the gland. A few data have also been obtained on the distribution of I^{131} in some of the major components of the egg within 2 hours after injection of radioiodide.

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MATERIAL AND METHODS

These studies were made on eggs from White Leghorn chickens. Incubation for the required interval was at 37.5° C. in a forced draft incubator. The eggs had been brought to room temperature before they were placed in the incubator, and no allowance was made for a "warm up" period; age was computed from the time that the eggs were placed in the incubator. The embryos corresponded to the stages described by Hamburger and Hamilton (1951). The thyroid gland morphology corresponded to the appropriate stages described by Venzke (1949).

Forty microcuries of I^{131} in the form of 0.05 ml. of carrier-free NaI was injected into one of the allantoic veins by means of a fine cannula sealed into a 0.1 ml. serological pipette. The window in the shell was sealed with "scotch" tape and the eggs were placed in a still air incubator until needed. At appropriate intervals they were reopened and a venous sample (0.05 ml.) was obtained and delivered into 0.95 ml. of H_2O containing 1.0 mg. of NaI. Immediately after the blood was taken the embryo was removed from the egg. Every effort was made to drain any extraneous fluids from the carcass. It is clear, from the values obtained for the I^{131} concentration in possible contaminating fluids (Table 2), that such contamination could not make an appreciable contribution to the concentration of I^{131} in the carcass. In no case was a carcass contaminated with yolk. Thyroid glands were dissected out, measured with an ocular micrometer and then placed into either Bouin's solution or an homogenizer containing 4 ml. of ice-cold H_2O to which 1.0 mg. of NaI carrier had been added. The weights of the thyroid glands were calculated by computing their volume from the lengths of their major axes and assuming a density of one. The carcass below the neck was weighed and put into a sample bottle or an homogenizer which contained 5 ml. ice-cold 8% TCA.

Complete serial sections were made of thyroid lobes from several 7-, 9- and 10-day embryos. Sections were stained with hematoxylin and eosin. Later the sections from 3 of the 7-day embryos and 4 of the 9-day embryos were destained and then treated with periodic acid-Schiff reagent to test for colloid.

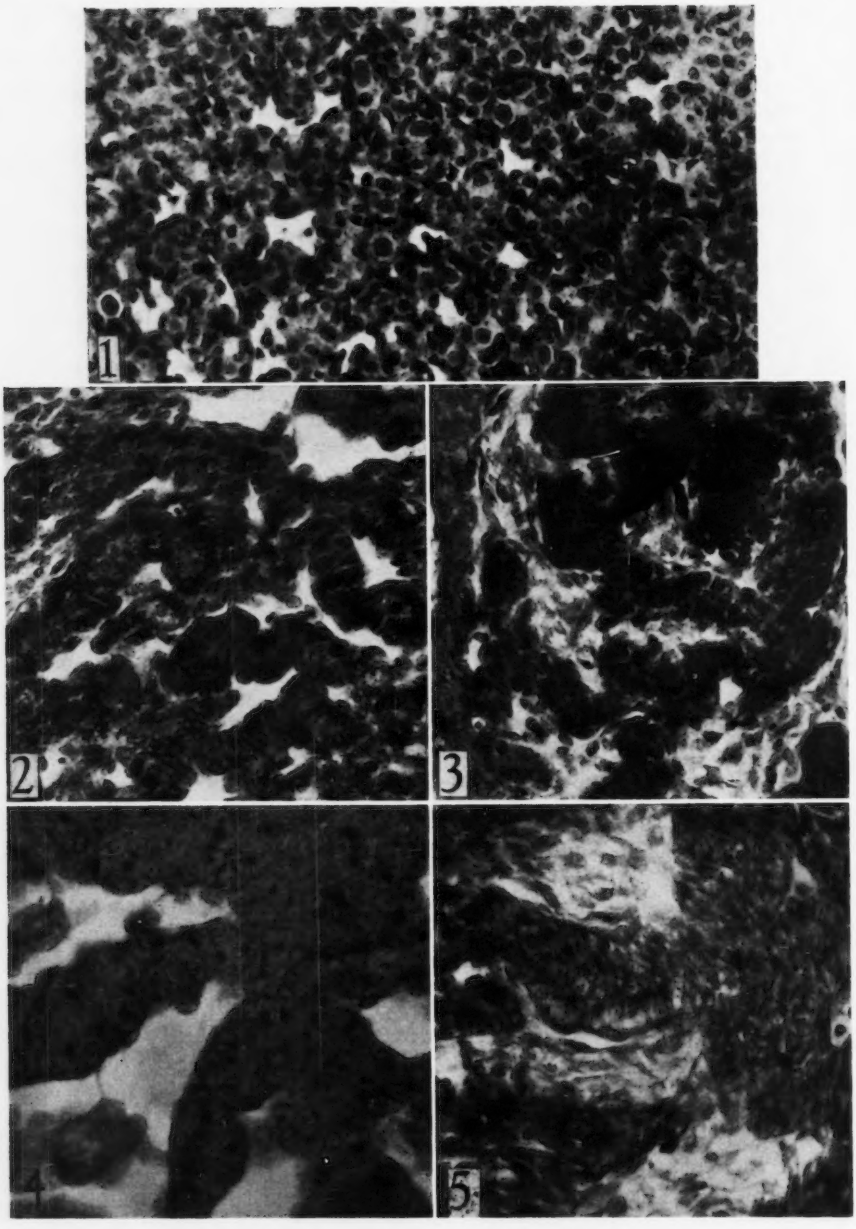
I^{131} in the diluted blood was determined by beta counting of a dried aliquot. Self absorption corrections were found to be negligible. Protein binding of I^{131} in the carcass was tested by homogenization in 8% TCA, centrifugation and determination of I^{131} in both the supernatant and the sediment by gamma counting. Protein binding of I^{131} in the thyroid glands was assessed after homogenization, deproteinization with the $Ba(OH)_2$-$ZnSO_4$ reagent of Somogyi (1945) and centrifugation.[2] I^{131} in the sediment was measured by gamma counting, that in the supernatant by beta or gamma counting. Some of the glands were dried and I^{131} was determined in them by beta counting. Although results using this technique agreed reasonably well with those using homogenized material only data from the latter are presented in the tables because of uncertainty of self-absorption corrections.

In a few 7-day eggs I^{131} determinations were made on allantoic fluid, amniotic fluid, blood, carcass below the head, fluid yolk and solid yolk. Samples for this survey were taken at approximately 75 minutes after injection.

RESULTS

The histology of the hematoxylin-eosin stained sections of the thyroid glands of the embryos studied (Figs. 1–3) appeared to duplicate observa-

[2] Under the conditions used Somogyi's reagent has been found to precipitate organic bound I^{131} more completely than does TCA, although the reagent does not precipitate an appreciable amount of radioiodide (Wollman and Scow, unpublished results).



FIGS. 1–5 THYROID GLANDS OF EMBRYONIC CHICKS

tions reported by others (Bradway, 1929; Hopkins, 1935; Venzke, 1949). The 10-day gland consisted of a fairly solid arrangement of epithelial cells and many droplets of colloid with a diameter one or two times that of a cell nucleus. The colloid stained a deeper pink than the cytoplasm. Both the 9-day and the 7-day glands consisted of epithelial cords separated by a loose mesenchyme. No colloid was visible. The periodic acid-Shiff preparations of the 9-day glands showed many deep magenta stained droplets of colloid in an otherwise pale pink section (Fig. 4). The diameter of these droplets was approximately one-half that of a nucleus. In the preparations of the 7-day sections not more than a few PAS positive droplets per gland were seen (Fig. 5).

The radioiodine content of the blood fell rapidly after injection in both the 7- and 9-day embryos. By 65 minutes after the injection, the I^{131} content of the blood of the 9-day embryos was not significantly above that expected from a uniform distribution of the I^{131} throughout the egg contents. The I^{131} content of the blood of the 7-day embryos was somewhat higher than that of the 9-day embryos. Although the variation from embryo to embryo was considerable there were indications in both age groups of a continuing decline of the I^{131} concentration in the blood during the experimental period.

In the 9-day embryos, a significant fraction of the injected I^{131} (average 0.26%) was found in the thyroid glands approximately one to four hours after the I^{131} was injected (Table 1). The total I^{131} content appeared to be constant during the time interval during which data were obtained, since the value at approximately 90 minutes after injection was 0.29% and at 200 minutes was 0.24%. In tests of four embryos, at least half the thyroid I^{131} was protein-bound 80 minutes after injection and at least 90% was bound after 3.5 hours. The concentration of radioiodine in these 9-day glands averaged over 5000 times that in blood, and increased significantly with increasing time after injection (Fig. 6). The concentration of non-precipitable I^{131} was only 200 to 800 times that of blood. In contrast, the

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EXPLANATION OF PLATE (FIGURES 1–5)

FIG. 1. Tenth day of incubation. H and E stain. Many colloid droplets present. ×340.

FIG. 2. Ninth day. H. and E. stain. ×340.

FIG. 3. Seventh day. H. and E. stain. ×340.

FIG. 4. Ninth day. Periodic acid-Schiff preparation. Note large number of PAS positive droplets. ×520.

FIG. 5. Seventh day. Periodic acid-Schiff preparation. Note absence of PAS positive droplets. ×520.

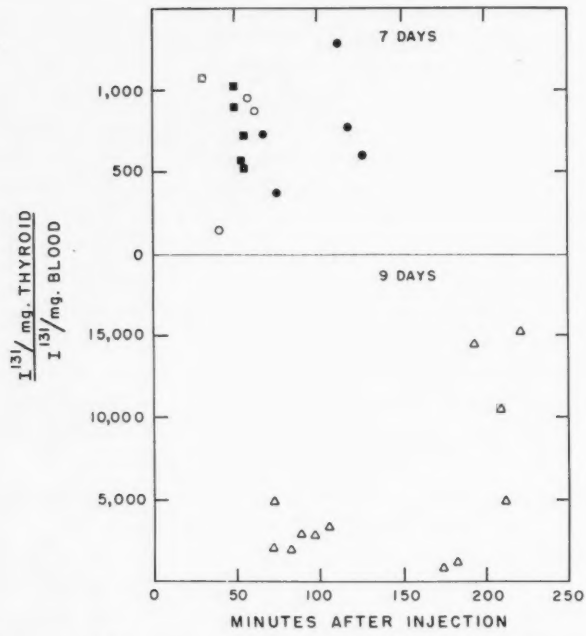

FIG. 6. The ratio of I^{131} content per mg. of thyroid gland to the I^{131} content per mg. of blood at various time intervals after injection of radioiodide. Data are shown for seven- and nine-day embryos. The three different symbols used in the seven-day data represent different experiments.

TABLE 1. THE RATIO OF I^{131} CONCENTRATION IN CARCASS AND THYROID GLAND TO I^{131} CONCENTRATION IN BLOOD OF CHICK EMBRYOS

Days incubated	Number of eggs	Time interval injection to sampling, minutes	Estimated thyroid weight, mg.	Average carcass weight,[1] gm.	Per cent dose in thyroid gland	I^{131}/mg. carcass / I^{131}/mg. blood	I^{131}/mg. thyroid / I^{131}/mg. blood	
							Total	Free
9	12	71 to 223	0.16	1.0	0.26 (12)	0.75 ± .04[2] (10)[3]	5400 ± 1200 (12)	400 ± 200 (3)
	6	30 to 65		0.23	0.024 to 0.11 (3)	0.95 ± 0.07 (5)	700 (4)	700 (4)
7	6	50	0.021	0.24	0.022 (5)	0.82 ± 0.05 (6)	700 (5)	700 (5)
	9	68 to 136		0.26	0.0037 to 0.068 (5)	0.80 ± 0.06 (9)	400 ± 106 (5)	400 ± 106 (5)

[1] Body below neck.
[2] Mean ± standard error.
[3] Number of eggs on which datum is based.

concentration of the carcass I^{131} averaged only 0.75 times that of blood and all of it was non-precipitable (Fig. 7).

In the 7-day embryos only .004 to .11% of the injected I^{131} was found in the thyroid gland. The radioiodine content was higher when the sampling was done relatively soon after the injection and lower when the sampling was delayed. No precipitable I^{131} was detected in the 7-day embryo thyroids. The ratio of the I^{131} content of the thyroid gland to the I^{131} content per mg. of blood was 15 ± 4. The concentration of I^{131} in these glands averaged 400 to 700 times that of blood (Fig. 6). The average value of the ratio was relatively constant during the time interval between 30 to 130 minutes after injection although measurements indicated that the I^{131} concentration of the blood at the end of this time interval could be only a few per cent of that at 30 minutes. The average concentration of I^{131} in the carcass varied from 0.80 to 0.95 times that of blood (Fig. 7) and was also independent of the time interval after injection. All the carcass I^{131} was non-precipitable.

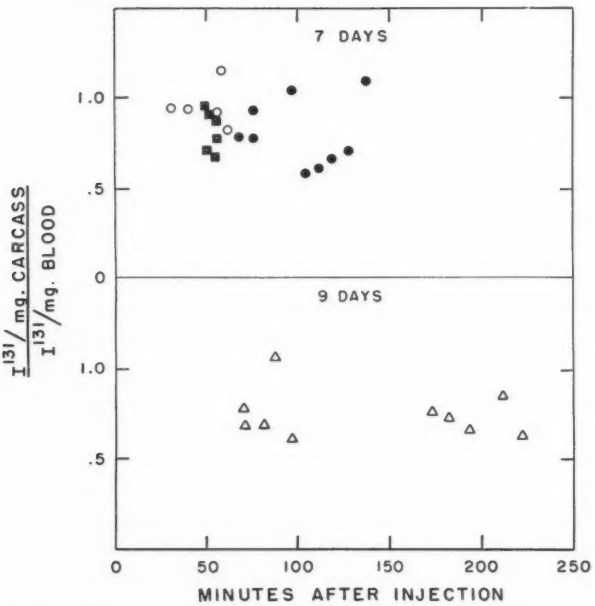

FIG. 7. The ratio of I^{131} content per mg. of carcass to the I^{131} content per mg. of blood at various time intervals after injection of radioiodide. Only the body below the neck was used in carcass determinations of these 7- and 9-day embryos. The three different symbols used in the seven-day data represent different experiments.

TABLE 2. DISTRIBUTION OF I^{131} AMONG SOME COMPONENTS OF THE CHICKEN EGG INCUBATED FOR SEVEN DAYS[1]

Component	Sample volume, ml.	Activity per ml., counts/sec/ml.	Total component volume,[2] ml.	Total activity in component, counts/sec.	Per cent dose in component	Activity per ml. / Activity per ml. if uniform distribution of I^{131}
Allantoic fluid	1.0	5,070 3,750	0.9	3,900	1.4	0.80
Amniotic fluid	0.05	1,670 1,870	1.3	2,300	0.80	0.33
Fluid yolk	1.0	14,900 13,500	13.0	185,000	65.0	2.6
Solid yolk	2.0	41 1,030	18.0	9,000	3.0	0.09
Head	0.406 0.473	1,930 1,520	0.41 0.47	785 716	0.26	0.32
Body	0.230 0.236	1,540 1,090	0.23 0.23	354 246	0.11	0.24
Total Egg		5,400	53.0	286,000	100.00	
Remainder of Egg[3]		4,500	19.0	85,000	29.7	0.83

[1] Approximately 75 minutes after injection of radioiodide.
[2] Volume of allantoic fluid and amniotic fluid from Romanoff and Hayward (1943). Volume of fluid yolk and solid yolk from Romanoff (1943).
[3] Data calculated by difference.

One of the striking findings in the initial experiments was that the concentration of I^{131} in the blood, carcass or allantoic fluid was less than would be expected if the injected I^{131} were uniformly distributed in the egg. In an exploration of a few 7-day eggs it was found (Table 2) that a major concentration of the I^{131}, both in regard to per cent of total dose and to relative activity occurred in the fluid yolk, which had ten times the concentration found in the carcass. The amniotic fluid had approximately the the same I^{131} concentration as the carcass while that of the allantoic fluid was somewhat higher. A number of components (e.g., yolk-sac membrane, albumen) were not tested and it is possible that a considerable concentration of I^{131} may have been present in one of these sites. For example, if it can be assumed that no I^{131} was present in the shell, the average I^{131} concentration in albumen and membranes would be half that in fluid yolk.

DISCUSSION

The difference in the results using 7-day and 9-day eggs cannot be attributed to either differences in the batches of fertile eggs used for the 7-

day and for the 9-day eggs, nor to differences in the composition of the stock radioiodide solution. The data listed in the last line of Table 1 and in solid circles in Figures 6 and 7 were obtained on eggs from the same batch as the 9-day eggs. Aliquots of the same stock radioiodide solution were used.

The data obtained in the present investigation show that the thyroid gland of the 7-day chick embryo has undergone biochemical differentiation to the extent that it already possesses the ability to maintain a concentration of non-precipitable I^{131} grossly elevated above that of the blood. The fact that the ratio of the concentrations of I^{131} in thyroid gland and blood was relatively constant while the I^{131} concentration of the blood was dropping suggests relatively free exchange and equilibration of the I^{131} in thyroid gland and blood. The capacity to incorporate iodide into organic molecules precipitable with Somogyi's reagent could not be demonstrated in these thyroid glands. If organic binding occurs it must be at a much slower rate than in the thyroid glands of the 9-day embryos which were found to have the capacity to incorporate radoiiodide into organic binding, in addition to the capacity to concentrate radiodide.

The above indications of biochemical differentiation both appear prior to the development of follicles in the chick embryo. It has been possible to demonstrate colloid-like material with the periodic acid-Schiff method in the stage in which organic binding of radioiodide was detected (9-day thyroids) and to demonstrate an almost complete absence of this material when radioiodide binding was not detected. In contrast, organic-bound iodine has been reported to occur prior to the appearance of both follicles and colloid in the thyroid gland of the fetal pig (Rankin, 1941) and the fetal calf (Koneff, Nichols, Wolff and Chaikoff, 1949).

There is an apparent contradiction between the present data demonstrating that the thyroid glands of the 9-day embryo have the capacity to bind iodide, and the work of Sun (1932), Hopkins (1935), and Hansborough and Khan (1951), who were not able to demonstrate organic bound iodine before the tenth or eleventh day of incubation. This contradiction may not be a real one. The present data demonstrate a qualitative capacity to incorporate available radioiodide into organic binding. There is no reason to believe that the radioiodide which was bound in the thyroid gland had equilibrated with endogenous iodide in the egg, or that endogenous iodide was present in the blood of the embryo and available for the thyroid gland to concentrate and bind. Sun's (1932) measurements on the total iodine concentration of the thyroid gland of the 9- and 10-day embryos when compared with the present data suggest that either endogenous egg iodine is not available to the embryonic thyroid gland or that the actual rate of binding of available iodide is quantitatively slow on the ninth day.

Hansborough and Khan's (1951) radioautograph studies (in which the radioiodide was injected into fertile eggs prior to incubation, and in which case it might be safe to assume that the radioiodide had equilibrated with endogenous iodide) suggest that the per cent of the endogenous iodide that is bound in the thyroid gland is too small to detect by their method prior to 11 days of incubation.[3]

No attempt has been made to determine the stage at which protein binding of radioiodide begins. This may be a difficult thing to do for, as shown by Wolff, Chaikoff and Nichols (1949), in the calf the concentration of bound thyroid iodine increases gradually but continuously throughout the period of fetal life which they studied. If this is so in general, the fixed sensitivity of radioautograph procedures as conventionally done may not be entirely satisfactory for determining onset of function, since if the sensitivity is fixed, i.e., if the dose of I^{131}, exposure time, and the sensitivity of the film are fixed, the measurements will probably give too late a time interval for onset of binding. For, if thyroid activity increases gradually during fetal life, increases in the sensitivity of the procedure might be expected to allow detection of a positive radioautograph at progressively earlier stages in development.

Removal of injected radioiodide from the blood and equilibration between the blood and the carcass were rapid. However, the concentration of I^{131} in the various parts of the egg was not uniform. In the 7-day embryos only the fluid yolk had a concentration greater than that expected from a uniform distribution of I^{131} throughout the egg contents. The concentration of I^{131} in the fluid yolk was ten times that of the blood, suggesting that the yolk-sac membrane has an active concentrating mechanism for iodide.

SUMMARY

Radioiodide was injected into an allantoic vein of 9-day and 7-day chick embryos. Quantitative determinations of I^{131} in thyroid gland, carcass, and blood were made using Geiger tubes. In a few embryos fluid and solid yolk, allantoic fluid and amniotic fluid were also assayed.

The thyroid glands of both the 9- and 7-day embryos concentrated

[3] After this manuscript was submitted for publication two papers became available to us which support and supplement these remarks. Stoll and Blanquet (Annales d'Endocrinologie, 13: 569. 1952) found that six hours after radioiodide was administered to a 7-day egg the I^{131} content per unit weight of the thyroid was higher than that of the heart. In contrast, Blanquet, Stoll and Capot (C. R. Soc. de Biol. 146: 1103. 1952) found that when radioiodide was administered to laying hens 100 per cent of the I^{131} in the 7-day egg was found in the yolk plus albumen. No I^{131} was found in the embryo until the eighth day, and none in the thyroid until the tenth day.

I^{131}. In 9-day thyroids, the I^{131} concentration averaged 5000 times that of blood, and most of this was organic bound. The I^{131} concentration in 7-day glands was 400 to 700 times that of the blood, and almost all appeared to be non-precipitable and freely exchangeable with blood I^{131}. I^{131} concentration in the carcass was about 0.7 to 0.9 times that in blood.

The fluid yolk accumulated the greater proportion (65%) of the total dose within a relatively short time (1.5 hours).

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EFFECTS OF ANDROGENS AND ESTROGENS ON β-GLUCURONIDASE IN INBRED MICE[1,2]

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IN RECENT years, Morrow *et al.* (1949, 1950) reported that certain pure inbred strains of mice exhibited strikingly low concentrations of β-glucuronidase in the liver, spleen and kidney. Cohen and Bittner (1951) found that mammary tissue β-glucuronidase activity bore a relationship to the strain of mice employed, extremely low concentrations being found in the C3H strain .

Among the interesting questions which these findings provoked is the one of whether or not tissue β-glucuronidase in these low-glucuronidase strains is responsive to extrinsic factors. It is known that certain glucuronidogenic drugs will produce an increase in β-glucuronidase activity in the non-sex organs (Fishman, 1940)) and that estrogen will restore uterine β-glucuronidase activity of the castrated mouse to normal levels Fishman and Fishman, 1944; Fishman, 1947) with no alteration in the non-sex organ glucuronidase. (Fishman, 1947; Harris and Cohen, 1951). An exception to this lack of effect in the non-sex organs was reported by Kerr, Campbell, and Levvy (1949) who found that estrone but not estriol or estradiol produced an increase in liver β-glucuronidase. In all of these experiments, mice of mixed strain were employed. Accordingly, investigation was undertaken to explore the effect of extrinsic factors with regard to organ β-glucuronidase activity in inbred mice.

In a previous paper, (Fishman, 1951) it was reported that stilbestrol stimulated liver but not kidney β-glucuronidase activity in C3H mice whereas testosterone and methylandrostenediol produced a striking increase in kidney but not in liver β-glucuronidase. The present paper represents essentially a systematic investigation of the effects of the estrogen and androgen in one low-β-glucuronidase mouse strain (C3H) and in one

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high-β-glucuronidase strain (129). In addition, new data are presented on tissue glucuronidase in a number of mouse strains.

METHODS AND EXPERIMENTAL PROCEDURES

Mice of pure inbred strain were obtained from the Jackson Memorial Laboratories, Bar Harbor, Maine and, in general, were arranged in groups of 5 to 6 animals for the experiments. These mice, weighing 20 ± 3 grams, were approximately two months old.

β-Glucuronidase activity was determined by the method of Fishman, Springer, and Brunetti (1948) with modifications (Fishman, Kasdon, and Homburger, 1950) employing acetate buffer homogenates of tissue. These were prepared by homogenizing a small amount of tissue (15–60 mgm.) in 10 cc. of 0.1 ml acetate buffer (pH 4.5). Duplicate specimens of 0.1 cc. of the well-shaken homogenate were taken for analysis. Following the hydrolysis of phenolphthalein glucuronic acid, the liberated phenolphthalein was measured colorimetrically. β-Glucuronidase activity has been expressed in the usual units per gram of wet tissue. (Fishman, Springer, and Brunetti, 1948). In the case of seminal vesicles and uteri, the secretions were expressed and the tissues dried with filter paper. These tissues were then weighed on a microtorsion balance and later homogenized and assayed as described above.

Mice of both sexes of the following strains were sacrificed by cervical fracture and determinations were made of the β-glucuronidase activity of liver, kidney, and spleen; C3H, AKR, CAF_1, BAF_1, DAH and 129.

In order to study the effects of sex hormones on tissue β-glucuronidase, mice of both sexes of the C3H and 129 strains were selected and arranged in suitable groups. Untreated groups received injections of peanut oil (0.1 ml.) every other day for 14 days to serve as controls to the experimental groups which received 500 micrograms of either stilbestrol or testosterone propionate or both in 0.1 ml. of peanut oil under the same dosage regime. In the castrated animals, a period of approximately 14 days was allowed to elapse before injections were begun. In all mice, β-glucuronidase determinations were performed on the fresh liver, kidney, and spleen. Assays of uterine and seminal vesicle β-glucuronidase were done in female and male animals respectively.

Experiments were performed to discover the effect of testosterone propionate on kidney β-glucuronidase in mice of several pure inbred strains and to estimate a dosage-response curve for two strains of mice.

RESULTS

Strain and sex differences. Of the six strains studied, four (BAF_1, CAF_1, 129, DBA) were found to be high-B-glucuronidase strains and two (C3H, AKR) low-β-glucuronidase strains (Table 1). The Bar Harbor AKR can now be characterized as a low-β-glucuronidase strain as well as one highly susceptible to spontaneous leukemia. The kidneys of male mice of the BAF_1, CAF_1, AKR and DBA strains assayed higher glucuronidase activities than did the kidneys of female mice in the corresponding strain. However, mice of the C3H and 129 strains showed no sex difference with respect to kidney β-glucuronidase.

Effects of castration on tissue β-glucuronidase activity are recorded in Table 2 along with the significance of the differences of the means (Table 3).

TABLE 1. A STUDY OF TISSUE β-GLUCURONIDASE IN RELATION TO STRAIN AND SEX

Strain	Sex	Tissue β-glucuronidase activity (Units/Gram)		
		Kidney (No.)	Spleen (No.)	Liver (No.)
C_3H	F	309 (5)	1391 (5)	485 (5)
	M	238 (5)	1156 (6)	479 (6)
AKR	F	449 (12)	1938 (12)	415 (12)
	M	671 (18)	1748 (18)	519 (18)
BAF_1	F	1829 (6)	4940 (6)	4545 (5)
	M	2655 (6)	4632 (6)	3134 (6)
CAF_1	F	1803 (6)	3660 (6)	3425 (6)
	M	3780 (6)	3548 (6)	4045 (6)
129	F	1376 (6)	7712 (6)	3187 (6)
	M	1460 (6)	6878 (5)	4050 (6)
dba	F	1547 (8)	4515 (8)	3936 (8)
	M	3192 (10)	4613 (9)	4278 (10)

In the 129 strain, castration of the females resulted in an elevation of liver and spleen β-glucuronidase and a decrease in uterine enzyme, whereas in the male sex, only the seminal vesicles underwent a change (increase). In the females of the C3H strain, castration caused no change in β-glucuronidase activity except for a questionable increase in kidney enzyme. The male C3H mice behaved in a similar fashion to the 129 animals, only seminal vesicle β-glucuronidase increasing.

Testosterone propionate (Tables 2 and 3) produced marked elevations in kidney β-glucuronidase in mice of both sexes, intact and castrate, of the 129 and C3H strains. Other responses common to both strains were the reduction in uterine β-glucuronidase of the intact mice and the decrease in seminal vesicle β-glucuronidase. Whereas, in the C_3H mice, liver and spleen enzyme levels remained essentially unchanged, in the 129 mice, the liver of the intact female and liver and spleen of castrate males showed an increase while the spleen of castrate females exhibited a decrease in their enzyme values.

Stilbestrol administration (Tables 2 and 3). Except for the castrate male C3H mice, no experimental groups showed a change in kidney β-glucuronidase activity. Other findings common to both strains were the reduction in seminal vesicle β-glucuronidase in intact male mice and the increased activity in the castrated males. Although liver β-glucuronidase was elevated only in intact females of the 129 strain, this increase was a uniform finding in all four experimental groups of C3H mice. Increases in spleen β-glucuronidase were observed in both sexes of 129 strain mice but only in the males (both intact and castrated) of C3H mice. Uterine β-glucuroni-

TABLE 2. COMPARISON OF β-GLUCURONIDASE DATA IN C3H AND 129 STRAINS OF MICE

Exp. No.	Strain	Sex	Condition	Tissue β-glucuronidase (units/gram)				
				Kidney	Liver	Spleen	Uterus	Seminal vesicles
			A. (CASTRATION)					
1	129	F	Intact	1,376 (6)	3,187 (6)	7,712 (6)	2,827 (6)	
2	129	F	Castrate	1,536 (9)	4,566 (9)	11,401 (9)	1,498 (9)	
3	129	M	Intact	1,460 (6)	4,050 (6)	6,878 (5)		3,937 (6)
4	129	M	Castrate	1,404 (10)	4,258 (10)	6,136 (10)		15,070 (7)
5	C3H	F	Intact	301 (5)	481 (5)	1,391 (5)	1,036 (5)	
6	C3H	F	Castrate	403 (6)	575 (6)	1,536 (6)	1,085 (6)	
7	C3H	M	Intact	287 (10)	506 (10)	1,156 (6)		91 (6)
8	C3H	M	Castrate	302 (6)	651 (6)	1,536 (6)		3,230 (6)
			B. (TESTOSTERONE PROPIONATE)					
9	129	F	Intact	5,895 (6)	4,290 (6)	5,994 (6)	1,942 (6)	
10	129	F	Castrate	6,546 (5)	4,084 (5)	6,674 (5)	1,393 (5)	
11	129	M	Intact	6,098 (6)	3,400 (6)	6,487 (6)		3,400 (6)
12	129	M	Castrate	14,230 (5)	5,802 (5)	10,888 (5)		4,378 (5)
13	C3H	F	Intact	3,121 (6)	630 (5)	1,307 (5)	402 (6)	
14	C3H	F	Castrate	1,991 (6)	664 (6)	1,401 (6)	638 (6)	
15	C3H	M	Intact	2,738 (5)	503 (5)	1,394 (5)		139 (5)
16	C3H	M	Castrate	2,940 (4)	606 (4)	1,629 (4)		260 (4)
			C. (STILBESTROL)					
17	129	F	Intact	1,658 (6)	6,845 (6)	17,208 (6)	4,148 (6)	
18	129	F	Castrate	1,538 (5)	5,068 (4)	9,795 (4)	4,018 (4)	
19	129	M	Intact	1,504 (6)	4,557 (6)	13,238 (6)		8,856 (5)
20	129	M	Castrate	1,420 (5)	4,806 (5)	11,406 (5)		7,593 (4)
21	C3H	F	Intact	305 (6)	930 (6)	1,403 (6)	1,043 (6)	
22	C3H	F	Castrate	365 (6)	1,018 (6)	2,136 (5)	1,165 (6)	
23	C3H	M	Intact	364 (6)	986 (6)	1,866 (6)		1,704 (6)
24	C3H	M	Castrate	448 (6)	1,369 (6)	2,425 (6)		1,795 (6)
			D. (TESTOSTERONE PROPIONATE+STILBESTROL)					
25	129	F	Intact	2,355 (5)	6,558 (5)	10,444 (5)	3,572 (5)	
26	129	F	Castrate	1,991 (5)	5,892 (5)	8,852 (5)	3,378 (5)	
27	129	M	Intact	3,042 (4)	6,222 (4)	8,712 (4)		3,327 (4)
28	129	M	Castrate	1,283 (6)	3,870 (6)	8,113 (6)		26,250 (4)
29	C3H	F	Intact	1,062 (5)	1,153 (5)	1,413 (5)	801 (5)	
30	C3H	F	Castrate	1,051 (5)	1,052 (6)	2,381 (6)	1,058 (6)	
31	C3H	M	Intact	1,625 (6)	947 (6)	1,732 (6)		275 (6)
32	C3H	M	Castrate	1,902 (6)	1,176 (6)	2,378 (6)		238 (4)

TABLE 3. STATISTICAL SIGNIFICANCE OF DIFFERENCES OF MEAN VALUES RECORDED IN TABLE II; t AND P VALUES

Comparison of the means Exp. No.	vs. Exp. No.	Organ				
		Kidney	Liver	Spleen	Uterus	Seminal vesicles
			A. (CASTRATION)			
1	2		2.5(0.03)	3.7(0.01)	3.7(0.01)	
3	4					13.8(0.01)
5	6					
7	8					20(0.01)
			B. (TESTOSTERONE PROPIONATE)			
9	1	6.5(0.01)	5.2(0.01)		1.9(0.1)	
10	2	12.4(0.01)		4.5(0.01)		
11	3	21.2(0.01)				
12	4	20.0(0.01)	3.5(0.01)	6.1(0.01)		13.5(0.01)
13	5	20.0(0.01)			2.3(0.05)	
14	6	5.0(0.01)			3.3(0.01)	
15	7	10.4(0.01)				
16	8	3.8(0.01)				20(0.01)
			C. (STILBESTROL)			
17	1		6.5(0.01)	21.0(0.01)	2.5(0.04)	
18	2				3.2(0.01)	
19	3			2.5(0.03)		9.0(0.01)
20	4			4.1(0.01)		4.0(0.01)
21	5		4.0(0.01)			
22	6		8.4(0.01)			
23	7		8.0(0.01)	3.3(0.01)		20(0.01)
24	8	3.8(0.01)	5.4(0.01)	4.8(0.01)		3.1(0.01)
			D. (TESTOSTERONE PROPIONATE+STILBESTROL)			
25	9	4.5(0.01)	7.5(0.01)	5.1(0.01)	4.4(0.01)	
25	17	3.4(0.01)		3.9(0.01)		
26	10	8.8(0.01)	8.1(0.01)		7.8(0.01)	
26	18					
27	11	3.2(0.01)	8.1(0.01)			
27	19			2.2(0.06)		
28	12	23.1(0.01)	6.8(0.01)			+
28	20					+
29	13	2.8(0.02)	5.4(0.01)		6.6(0.01)	
29	21	4.8(0.01)				
30	14	2.9(0.02)	5.1(0.01)	5.1(0.01)	6.8(0.01)	3.5(0.01)
30	22	15.0(0.01)				
31	15	2.8(0.02)	4.4(0.01)			4.2(0.01)
31	23	4.2(0.01)				14.8(0.01)
32	16	1.3(0.2)	4.7(0.01)	6.4(0.01)		
32	24	4.6(0.01)				13.2(0.01)

Statistical studies were done only where the differences between the means were greater than 33% of the lesser value. This accounts for the blank spaces in the table.

dase was raised in 129 intact and castrate females but was not changed in the corresponding groups of the C3H strain.

Testosterone propionate plus stilbestrol (Tables 2 and 3) Androgen and estrogen exhibited neutralizing actions on each other in some organs but not in others depending on the strain, the sex and the presence or absence of the gonads. Thus, estrogen, sometimes partially and on other occasions

wholly, prevented the characteristic elevation of kidney β-glucuronidase by testosterone propionate in both strains. However, the androgen did not prevent (except for experiment 28) the estrogen-induced increases in liver β-glucuronidase of C3H (experiments 29, 30, 31, 32) and 129 (experiment 25) mice or affect the liver enzyme values (experiments 26, 27 and 28). In the spleen, androgen counteracted the enzyme-enhancing effect of estrogen only in intact male and female 129 mice, while, in the uterus, androgen effected little or no change in the β-glucuronidase activity when compared with the values seen in the groups given estrogen alone. Seminal vesicle β-glucuronidase maintained levels similar to those of corresponding groups given androgen alone in all experiments except number 28.

Dosage-response curves. Data relating kidney β-glucuronidase to dosage of testosterone propionate in C3H and A Cloudman male mice appear in Figure 1. Evidently, testosterone propionate in physiological amounts is capable of causing a significant response in kidney β-glucuronidase.

Kidney glucuronidase response to testosterone in various strains of mice. The kidney β-glucuronidase response to testosterone propionate differs with the strain of animal employed (Table 4). Thus, two levels of stimulation were observed. The 129 and DBA strains exhibit a 4 to 5-fold increase

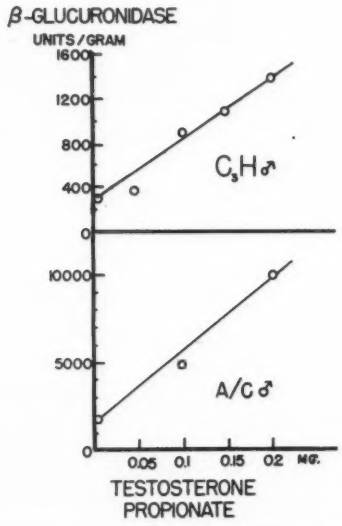

FIG. 1. Testosterone propionate dose-response curve. Each experimental point is the average of the β-glucuronidase activities of the kidneys of each group of 6 male mice. The daily dosage of testosterone propionate is indicated in the figure and the length of the experiment was 14 days.

and the C3H, AKR, CAF_1, BAF_1, and A Cloudman, a 9.5 to 12.5-fold elevation. It may be noteworthy that both low-β-glucuronidase strains fall into the second category.

TABLE 4. KIDNEY β-GLUCURONIDASE RESPONSE TO TESTOSTERONE PROPIONATE IN INBRED MICE (MALE)

Strain	Control animals	Experimental animals	Percentage increase
129	1,460 (6)	6,098 (6)	416
C3H	287 (10)	2,738 (5)	955
AKR	650 (6)	5,875 (6)	1,110
DBA	2,821 (10)	14,214 (7)	505
A Cloudman	3,396 (6)	42,075 (4)	1,245
BAF_1	2,655 (6)	26,300 (7)	990
CAF_1	3,780 (6)	25,450 (7)	675

DISCUSSION

In this study a new low β-glucuronidase strain (Bar Harbor AKR) has been discovered. At present, the following strains are known to belong to this category, C3H (Heston, Bittner, Andervont) DBA (Andervont). Law, Morrow and Greenspan (1952) have reported that the inheritance of the low liver β-glucuronidase in C3H mice is a characteristic of a single recessive gene. Although strain differences have been reported for a few other enzymes, this phenomenon has been studied systematically only with regard to the inheritance of β-glucuronidase. It would appear, therefore, that in mice, the genetic background of the animal is an important factor in determining tissue β-glucuronidase activity.

It has now been established, confirming our previous data (Fishman, 1951) that sex hormones have the ability of increasing the β-glucuronidase activity of certain tissues in mice of both a low- and a high-β-glucuronidase strain.

Androgen

In the case of androgen (testosterone propionate), it was possible to increase regularly the kidney β-glucuronidase to levels far above the highest values encountered in any untreated normal mice. This phenomenon has been demonstrated in both C3H and 129 strains of mice (Table 2) and in five other strains studied. In the 129 strain, on several occasions, β-glucuronidase elevations were seen also in liver and spleen, such changes being absent in corresponding groups of C_3H mice. Testosterone propionate prevented the characteristic rise in β-glucuronidase of the seminal vesicles which ordinarily follows the castration of males of both strains.

Estrogen

The action of estrogen (stilbestrol) on the other hand, differed from androgen in that kidney β-glucuronidase was essentially unaffected, and

liver and spleen β-glucuronidase showed significant increases, the extent of which depended on the strain and condition of the animals. However, in no experiment in C3H mice was it possible to increase liver β-glucuronidase to the levels found in high β-glucuronidase strains. This finding contrasts with the behavior of kidney β-glucuronidase in testosterone-treated low-glucuronidase strains. Seminal vesicle β-glucuronidase was increased by stilbestrol. The experiments in Table 2 were all repeated using 5 γ instead of 500 γ of estrogen and the corresponding results were essentially identical except that, in the 129 strain, spleen β-glucuronidase was in the range of the corresponding untreated control animals and the liver β-glucuronidase of C_3H mice did noot increase.

Testosterone-propionate and stilbestrol

In these experiments, stilbestrol always counteracted the action of androgen on kidney β-glucuronidase but androgen had no antagonistic action towards stilbestrol's action on liver, and only occasionally, on spleen β-glucuronidase. The data dealing with the uterus show a domination of the estrogen effects and those concerning seminal vesicles, except for experiment 28, resemble closely the results with androgen alone.

Another point of interest is the following strain difference: the antagonistic effect of estrogen on androgen-increased kidney β-glucuronidase is more marked in the 129 mice than it is in the C3H strain.

It appears that kidney β-glucuronidase is more specifically influenced by androgen and estrogen than any of the other tissues studied. It is also clear from other unpublished experiments dealing with borneol administration that the increase found in liver β-glucuronidase is by no means specific for stilbestrol. The possibility noted here of strain and sex differences in the ability of the liver to increase its β-glucuronidase activity with estrogen stimulation may explain the discrepancy in the reports of Kerr, Campbell and Levvy (1949) and Fishman (1947).

Castration effects

Clear-cut strain and sex differences have been demonstrated in the experiments concerned with the castration of 129 and C3H mice. Thus, castrated females of the 129 strain exhibit significant increases in liver and spleen β-glucuronidase which are not seen in the corresponding experiments with C3H mice; castrated males of the same strain do not show this increase. Castrated females of 129 mice (as has been reported for White Swiss animals (Fishman and Fishman, 1944; Fishman, 1947) show a significant decrease in uterine β-glucuronidase which is not seen in C3H castrate female mice. On the other hand, castrate male mice of both strains show a marked rise in the β-glucuronidase activity of the seminal vesicles.

Dosage-response curves

As seen from the dosage-response curves, significant elevations in kidney β-glucuronidase activity were obtained with amounts of testosterone propionate as little as 0.1 mg. per day. The maintenance of the weight of the seminal vesicles has required as much as 1 mg. per day. Accordingly, on the basis of these minimal effective doses, it may be stated that the kidney β-glucuronidase response to androgen is a physiological phenomenon.

Enzyme response to androgen in various strains

The comparison of the kidney β-glucuronidase response to testosterone propionate in inbred mouse strains indicates real quantitative differences ranging from 4 to 13-fold increments. As the only variable which is evident is the strain of the animal in these experiments, it is reasonable to assume that the β-glucuronidase response to testosterone is strain-dependent. If it is correct to assume that this enzyme phenomenon is an important aspect of androgen action in the kidney, the fundamental question follows as to whether or not sex hormone effects in general are influenced to an important degree by heredity.

CONCLUSIONS

1. A new low-β-glucuronidase mouse strain (AKR) has been found; liver, kidney and spleen exhibiting enzyme activity in the range observed for previously reported low-β-glucuronidase strains of inbred mice.

2. A comparison was made in one high- (129) and in one low- (C_3H) β-glucuronidase strain of the effects on tissue β-glucuronidase of castration and the administration of stilbestrol and testosterone propionate, alone and in combination.

3. Testosterone propionate produced marked elevations in kidney β-glucuronidase in mice of both sexes, intact and castrate, of the 129 and C3H strains. This action was neutralized usually in part and sometimes completely by simultaneously administered stilbestrol.

4. Stilbestrol increased the activity of liver β-glucuronidase in C3H mice, although to a much lower level than was characteristic of the liver values in the high-glucuronidase strains.

5. Following castration of male mice of 129 and C3H strains, seminal vesicle β-glucuronidase increased. Testosterone propionate neutralized this action.

6. From dosage-response curves completed in two strains of mice (C3H, A Cloudman), it appears that kidney β-glucuronidase responded significantly to 1.4 mg. of testosterone propionate given in divided doses over 14 days.

7. The response of kidney β-glucuronidase to testosterone propionate differed with the strain of mice employed.

Acknowledgments

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EXTENDED SURVIVAL OF ADRENALECTOMIZED RATS POSSESSING EAR- OR SPLEEN-GRAFTED OVARIES[1,2]

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IN MOST mammals removal of the adrenal glands results in deleterious changes leading to loss of body weight and terminating in death after a variable, usually brief, period of time. The female ground squirrel (*Citellus tridecemlineatus*) is apparently an exception to this rule since it lives for periods up to one year following adrenalectomy (Groat, 1943). Its ovaries are altered to the extent that some ovarian cells are described as "adrenocortical-like" and it is this altered histology that is held to be accountable for increased survival.

If an ovary is grafted to the ear of a castrate mouse or rat it becomes morphologically and functionally different from control ovaries in that it possesses thecal luteinization and it secretes an androgen (Hill, 1937a, 1937b; Deanesly, 1938). An ovary transplanted to the spleen of a rat becomes markedly luteinized, even to the point of luteoma formation, after a period of time (Biskind and Biskind, 1944). These two technics for producing deviations from normal ovarian structure and function have been used to study the possibility that transplanted ovaries, like the intact ones of the ground squirrel, may protect adrenalectomized animals against death from adrenal insufficiency. Hill (1948, 1949) demonstrated that ovaries grafted to the ears or spleen of mice can extend survival time following adrenalectomy (averages, 121–137 days as compared to 6.7–12.0 days for adrenalectomized controls). The present experiment indicates that grafted ovaries can extend survival following adrenalectomy in the albino rat as well and that ovaries grafted when immature are as effective as mature ovaries.

MATERIALS AND METHODS

200 male and female albino rats of the Wistar strain were used in this experiment. 189 of these were divided into four groups:

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[2] Portions of this material were presented in theses in partial fulfillment of the requirements for the degree of Master of Science (I.J.L. and M.H.G.).

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Group I. Adrenalectomized controls. 16 mature males and 40 mature females (weight 38–170 gm.) were subjected to no operative procedures before adrenalectomy.

Group II. Sham-operated adrenalectomized controls. 20 males and 17 females at the age of 21 days (weight 20–42 gm.) were castrated and implanted with a piece of uterus the size of an immature ovary into the spleen.

Group III. Ear-implanted experimentals. 29 mature females (weight 40–170 gm.) were castrated and autoplastically implanted with ovaries into the ears.

Group IV. Spleen-implanted experimentals. 27 males and 29 females at the age of 21 days (weight 19–46 gm.) and 11 mature females (40–170 gm.) were castrated. The females were then autoplastically implanted with an ovary into the spleen and the males were implanted with an ovary from a littermate female into the spleen.

All animals of all groups were bilaterally adrenalectomized 2–4 weeks after previous operations or when they matured. All animals were maintained on Purina laboratory chow and tap water, both *ad libitum*. The rats were weighed every 4 days following adrenalectomy, and they were examined daily to determine survival. The body weights and survival times in days following adrenalectomy were recorded for rats found dead. Since most control rats died within 25 days we arbitrarily considered as survivors only those rats that lived at least 3 times that long (75 days). Rats surviving 75 days or more were examined for presence of grossly visible adrenal regrowths and rests in the abdominal cavity, especially in the area of the kidneys. Reproductive function of 5 ear-implanted and 3 spleen-implanted mature females which had survived 168 days after adrenalectomy was studied by observing vaginal smears and copulation plugs. Standard histological technics were employed to study the structure of the grafts and suspected adrenal regrowths and rests.

Those experimental rats surviving more than 75 days were subjected to a standardized stress test (Marmorston-Gottesman and Gottesman, 1928) consisting of intraperitoneal injections of 15 mg. per 100 gm. body weight of histamine diphosphate. Under the conditions of this test intact rats survive and completely adrenalectomized rats die within 24 hours. Six intact and 5 newly adrenalectomized (3–4 days postoperative) rats were used as controls. By the use of the test it was hoped that the survival of rats possessing ovarian grafts could be compared with that of intact and adrenalectomized controls for the purpose of determining the adrenocortical-like protective action of the ovarian grafts. The time of death was noted for those rats which died within 24 hours after the histamine injection. Death within 24 hours was considered evidence for the presence of adrenocortical insufficiency.

RESULTS

Survival. The results regarding survival are summarized in Table 1. Group I (adrenalectomized controls). 8 out of 56 rats which lived longer than 75 days (range, 102–168 days) were found to possess large adrenal regrowths and hence were eliminated from consideration. 45 out of the remaining 48 adrenalectomized rats died within 23 days after adrenalectomy (mean, 13.7 ± 0.6 days; range, 4–23 days) and one died on the 53rd day. Two rats of this group survived 88 and 95 days; no adrenal tissue could be found to account for their prolonged survival. As tested by the χ^2 method, no significant differences in survival were found between males and females (see Table 2).

TABLE 1. SUMMARY OF SURVIVAL DATA

Group	No. of rats	No. eliminated for regrowths	No. considered	No. surviving 75 days or more	No. surviving 30 days or less
Adrenalectomized controls	56	8	48	2	45[1]
Sham-operated adrenalectomized controls	37	2	35	3	32
Ear-implanted experimentals	21	1	20	4	16
Spleen-implanted experimentals	67	2	65	18	42[2]

[1] One rat died 53 days after adrenalectomy.
[2] Five rats died 35, 50, 53, 55, and 56 days after adrenalectomy.

Group II (sham-operated adrenalectomized controls). 32 out of 37 adrenalectomized rats each bearing a uterine graft in the spleen died within 18 days after adrenalectomy (mean, 11.3 ± 0.5 days; range, 5–18 days). Two rats were eliminated because of adrenal regrowths. The remaining 3 rats of this group survived 76, 78, and 113 days after adrenalectomy and were then killed for autopsy. No significant differences in survival were found to exist between males and females of this group.

Since the χ^2 test revealed no significant difference in survival between rats of Groups I and II, the results of both control groups may be combined: 5 out of 83 combined controls survived 75 days following bilateral adrenalectomy.

Group III (ear-implanted experimentals). 4 out of 21 rats of this group had survived up to the time they were killed on the 168th day after adrenalectomy. A 5th survivor possessed a large adrenal regrowth and was eliminated from the results. The other 16 rats died within 30 days (mean, 13.6 ± 1.5 days; range, 7–29 days). We shall define a survival ratio (a/d) as the ratio of the number of rats which survive at least 75 days after adrenalectomy, a, to those which survive less than 30 days, d. For this group it is 4/16, which is significantly greater than that of the combined controls (5/77).

TABLE 2. STATISTICAL ANALYSIS OF SURVIVAL DATA

Hypothesis	X^2	p
Survival ratio* is independent of:		
1. Sex of adrenalectomized controls	.979	$> .30$
2. Sex of sham-operated adrenalectomized controls	.580	$> .30$
3. Sex of immature spleen-implanted experimentals	.164	$> .50$
4. Site of graft: ear or spleen	.752	$> .30$
5. Presence of uterine (sham) grafts in adrenalectomized controls	.653	$> .30$
6. Presence of ovarian grafts	13.355	$< .01$**

* Survival ratio is the ratio of the number of rats surviving 75 days or longer to those surviving less than 30 days after adrenalectomy.
** Hypothesis is rejected at the 1% level of confidence. The 5 preceding hypotheses are accepted.

Group IV (spleen-implanted experimentals). 2 out of 67 rats possessing ovarian implants in the spleen survived more than 75 days after adrenalectomy but were eliminated from consideration because they possessed large adrenal regrowths. 18 of the remaining 65 spleen-implanted rats survived at least 75 days after adrenalectomy (maximum, 168 days) before being killed and were found to possess no adrenal rests or regrowths. 42 of the remaining 47 rats died within 30 days (mean, 14.7 ± 0.7 days; range, 8–30 days) and 5 rats of this group died 35, 50, 53, 55, and 56 days following adrenalectomy. No significant survival differences were found between males and females, or between rats with mature and immature ovarian grafts. The ratio (a/d) of the number of rats which survived at least 75 days after adrenalectomy, a, to those which survived less than 30 days, d, is significantly higher for this experimental group, 18/42, than for the controls, 5/77.

Statistical analysis shows that no significant survival difference exists between the rats with ovaries grafted to the ears and those with ovarian grafts to the spleen. Thus the combined survival ratio for groups III and IV is 22/58.

Body weight. The body weights of all rats which survived 30 days or less showed similar rates of decline. Death from adrenocortical insufficiency was consistently preceded by a precipitous drop in body weight. In general those rats which survived 75 days or longer showed parallel weight gains regardless of whether they had received ovarian or uterine grafts or none at all.

Adrenal regrowths and rests. Thirteen rats which had survived 75 days were found to possess gross adrenal regrowths and had to be eliminated from the survival results (see above). These regrowths appeared at the usual site of the adrenal and sometimes reached normal adrenal size. Histological examination of the regrowths showed that cords of adrenocortical cells differentiated from remnants of the adrenal capsule. In no cases were adrenocortical rests as such found in any rats.

Ovarian function. The 4 mature female ear-grafted rats which survived adrenalectomy were examined for evidence of estrous cycles and mating behavior. Vaginal smears over a 20-day period showed that all of the rats with ovaries grafted to the ears showed normal 4–5 day estrous cycles. When in estrus these rats were observed to mate readily with normal males. Presence of copulation plugs confirmed that mating had occurred. The rats with ovaries grafted to the spleen showed no evidence of estrous cycles, nor would they mate.

Histology. Only spleen-grafted ovaries were examined in sufficient numbers to permit adequate histological analysis. Ovarian grafts from animals which survived for a significant period of time after adrenalectomy could

not consistently be distinguished from grafts taken from rats which died within the control period. In general, the histology of these ovaries was similar to that of the grafted ovaries described by Biskind and Biskind (1944). Ovaries from both groups varied in size from 2 to 10 mm. in diameter and some had follicles in all stages of development, with and without ova, and some follicles were cystic. All ovarian grafts were heavily luteinized or had infiltration of connective tissue into the corpora lutea. The ovarian grafts from animals that failed to survive usually were organized into discrete, normal-appearing corpora lutea, often surrounded by scanty connective tissue stroma. On the other hand the ovaries of rats which survived beyond the control period showed evidence of thecal luteinization and possessed islands of luteal tissue not organized into corpora lutea. These ovaries often appeared to be disorganized and con-

TABLE 3. RESULTS OF THE HISTAMINE TEST FOR ADRENOCORTICAL FUNCTION

Group	No. of rats	No. of rats surviving <24 hrs. after histamine	No. of rats surviving >24 hrs. after histamine
Intact rats	6	0	6
Newly adrenalectomized rats (3–4 days before histamine injection)	5	5	0
Adrenalectomized controls	3	0	3
Sham-operated adrenalectomized controls	3	3	0
Ear-implanted experimentals	4	0	4
Spleen-implanted experimentals	14	9	5

tained relatively generous amounts of connective tissue stroma. The corpora lutea of these ovaries were rarely normal in appearance due to presence of non-luteal cells interspersed among luteal cells, replacement of the centers by connective tissue, or complete absence of typical luteal cells. In no case did rats possessing ovaries without these histological features survive beyond the control period.

Histamine tests. Table 3 summarizes the results of the histamine tests. Three male and 2 female rats adrenalectomized 3–4 days before injection of histamine became prostrate and succumbed within an hour after injection. Three intact males and 3 intact females all regained usual activity within 2 hours and survived an observation period of 24 hours. The ability of spleen-grafted ovaries to prolong survival times of adrenalectomized rats did not necessarily equip them to withstand histamine stress. This is seen in the fact that 9 out of 14 of the rats which received ovarian grafts to the spleen failed to survive 24 hours after injection.

DISCUSSION

The data presented here indicate that rat ovaries, transplanted to the ears or spleen of albino rats, protect a significant proportion of such rats against weight loss and death from adrenocortical insufficiency for 75 days or longer following adrenalectomy. These results corroborate those of Hill (1948) who observed that out of a total of 105 mice with ovaries grafted to the ears, 52 survived beyond the 29 day limit for adrenalectomized controls. In the present experiment 20% of the ear-implanted rats survived more than 75 days after adrenalectomy, whereas survival was 49.1% for Hill's ear-implanted mice. In mice with ovaries grafted to the spleen, Hill (1949) found that 32 out of 47 females, or 68.1%, survived variable periods beyond 30 days. Survival beyond 75 days following adrenalectomy for our spleen-implanted rats was 27.7% compared to 68.1% survival beyond 30 days for Hill's spleen-implanted mice. It is difficult to explain these species differences; however it should be emphasized that these figures represent more agreement than they seem to show since Hill did not apply the criterion that animals must live at least 75 days after adrenalectomy in order to be counted as survivors.

The survival-prolonging function of rat ovaries is apparently independent of other ovarian functions. Since ovaries of immature rats afford protection against death from adrenocortical insufficiency equal to that of ovaries of adults, the present data eliminate maturation and estrous cycling of ovaries previous to transplantation as a necessary condition for prolongation of survival time.

Deanesly (1938) described a process of "thecal luteinization" in ovaries grafted to the ears of castrated male rats. She attributed the observed androgenic activity of such ovaries to their state of thecal luteinization. In the present experiment, ovaries in rats whose survival exceeded 75 days after adrenalectomy possessed extensive luteinization beyond the limits of any corpus luteum. Many of the rats which died within the control period had ovaries with a large amount of luteal tissue but in all cases it was confined to the limits of corpora lutea. It is likely that this difference in organization of the luteal tissue in the two groups is associated with differences in survival. Very possibly the cells of the ground squirrel ovary which Groat (1943) described as "adrenocortical-like" were, in fact, luteal cells.

It is not understood why survival was not extended for all adrenalectomized mice and rats possessing ovarian transplants. However not too much should be expected of grafted ovaries since even adrenals transplanted to similar sites offer only partial effectiveness with respect to life-prolongation (Stolpe and Schreiber, 1952).

Komrad and Wyman (1950) found that adrenal glands auto-transplanted to the dorsal musculature could protect all their rats against injection of histamine (in the same form and dosage as in the present experiment) after a minimum of 7 days following transplantation. However, more than half of our rats with spleen-grafted ovaries did not survive injection of histamine, although all 4 rats with ear-grafted ovaries did survive. Stolpe and Schreiber (1952) found that when adrenal glands were auto-transplanted to the same sites, spleen or ears, recipient rats survived histamine stress no better than rats with ovarian transplants unless sufficient exogenous ACTH was previously administered. It may be possible that grafted ovaries require stimulation by an anterior pituitary hormone in order to exercise full protective action against histamine stress.

Two hypotheses suggest themselves in explanation of the mechanism of the life-prolonging action of grafted ovaries. The first is that the grafted ovaries, in consequence of their ectopic position, secrete one or more substances qualitatively different from other ovarian secretions and life-prolonging in action. A precedent for this supposition is the androgenic function of ear-grafted ovaries in mice and rats (Hill, 1937a, 1937b; Deanesly, 1938; Lampton and Miller, 1940; Hernandez, 1943).

The second hypothesis is that the grafted ovaries, so highly luteinized, secrete sufficient progesterone to account for increased survival after adrenalectomy. Much evidence, direct and indirect, has indicated that progesterone is able to increase survival of rats for rather short periods of time following adrenalectomy (Gaunt, Nelson and Loomis, 1938; Greene, Wells and Ivy, 1939; Emery and Greco, 1940). However, experiments in our laboratory demonstrate that progesterone is ineffective in prolonging survival of rats more than 2 months after complete removal of adrenocortical tissue. For this reason, the first hypothesis is preferred.

SUMMARY

Twenty adult female Wistar rats were castrated and autoplastically implanted with an ovary into each ear. Eleven adult females were castrated and autoplastically implanted with an ovary into the spleen. Fifty-four 21-day old males and females were also castrated and auto- or homoplastically implanted with an immature ovary into the spleen. These rats were adrenalectomized 2 to 4 weeks postoperatively, or when mature. Forty-eight adult and immature adrenalectomized rats and 35 immature castrated sham-implanted adrenalectomized rats served as controls.

Seventy-seven out of 83 control rats died within 30 days after adrenalectomy, and 5 survived 75 days or longer. Four out of 20 ear-implanted and 18 out of 65 spleen-implanted experimental rats survived 75 days or longer,

a significantly larger proportion than among the controls. There were no differences in survival ratio between the sexes, between mature and immature rats, or between ear- and spleen-implanted rats.

Ovarian grafts from surviving rats possessed abundant luteal tissue, much of it not organized within corpora lutea, whereas the ovarian grafts from animals which died within the control period had luteal tissue mostly restricted to corpora lutea.

The ear-grafted ovaries supported normal ovarian cycling and mating behavior and protected against death from histamine poisoning. The spleen-grafted ovaries exhibited no cyclic behavior and protected only 9 out of 14 surviving rats against death from histamine poisoning.

It is hypothesized that ear- and spleen-grafted ovaries, in consequence of their ectopic position, secrete one or more unknown substances which have a life-prolonging action for adrenalectomized rats.

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THE EFFECT OF GROWTH HORMONE ON TIBIA GLYCOGEN CONTENT OF THE HYPOPHYSECTOMIZED RAT[1]

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GLYCOGEN has been implicated in endochondral calcification by Gutman and Yü, (1950) in their studies on the effect of several specific inhibitors on the enzymes of the glycolytic system. Moreover, Marks and Shorr (1950) have shown that a marked diminution of the *in vitro* calcification of cartilage slices occurs after a preliminary amolytic digestion of the glycogen present in epiphyseal cartilage. Since in the immature hypophysectomized rat ossification is markedly retarded, it was considered of interest to determine the effect of hypophysectomy on the glycogen content of actively growing bone. Furthermore, since the administration of growth hormone reactivates osteogenesis in immature hypophysectomized rats, a study was made of the effects of growth hormone treatment on the glycogen content of bone.

METHODS

Female rats of the Long-Evans strain were used. Normal animals twenty-one days and older had unrestricted access to Diet XIV; hypophysectomized animals were given Diet XIV and lettuce, supplemented with wet Diet I.[2]

For the determination of glycogen, the following procedure was employed. The left tibia of the rat was removed under amytal anaesthesia and rapidly cleaned of muscle and connective tissues. The fibula was cut off flush with the surface of tibia and discarded. After cutting the tibia in half both length and cross wise, the tibia was placed in 2 ml. hot 30% KOH and digested for 2 hours in a boiling water bath. The centrifuged digest was then transferred into a test tube graduated at 3 ml. volume and the bone residue remaining was washed twice with ½ ml. portions of 30% KOH. To the 3 ml. volume of the digest, 0.3 ml. 5% $NaSO_4$ was added followed by 4 ml. absolute ethanol. After mixing and bringing to a momentary boil, the tubes were chilled for at least 2 hours at 5° C. The precipitate was then centrifuged and the supernatant fluid decanted.

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[1] Aided by grants from the U. S. Public Health Service RG-409 and C-1098.

[2] Diet XIV consists of ground whole wheat 67.5%, casein 5%, alfalfa leaf meal 10%, fish meal 10%, fish oil 5%, NaCl 1.5%. Diet I consists of ground whole wheat 67.5%, casein 15%, skim milk powder, 7.5%, NaCl 0.75%, $CaCO_3$ 1.5%, melted fat 6.75%, fish oil 1.0%.

The precipitate was washed once with 70% and then 95% ethanol and dried. After dissolving the precipitate in either 3 or 5 ml. of water, depending on the quantity of glycogen expected, 0.8 ml. aliquots were removed for analysis by a modification of the anthrone method (Viles and Silvermann, 1949). The 0.8 ml. aliquots were delivered into 3 ml. glass stoppered volumetric flasks containing magnetically stirred "fleas." The flasks were then chilled in a crushed ice bath. With the "flea" stirring vigorously, 2.0 ml. of anthrone reagent (0.15% in 95% H_2SO_4) was added rapidly and stirred exactly one minute. The volumetric flasks were then transferred to a boiling water bath for exactly 7 minutes at which point maximum color development occurs under the conditions here employed. The flasks were then chilled rapidly in the crushed ice bath and diluted to volume with 2.5:1 H_2SO_4:H_2O. Optical density was measured at 625 mμ. Standards of twice reprecipitated glycogen were analyzed with each series of samples, since color development varies slightly with the age of the anthrone reagent. Analyses of known solutions of glycogen average 98% recovery by this method.

Solutions of purified chondroitin sulfate react with anthrone reagent to give an absorption curve with a maximum at 625μ. The presence of chondroitin sulfate in cartilage presented the possibility of interference in the determination of glycogen. This was ruled out by digestion of purified solutions of chondroitin sulfate in 30% KOH for 2 hours at boiling water bath temperature, after which time virtually no color development occurs. Furthermore, digestion of the glycogen obtained from bone with salivary amylase and analysis according to the above procedure resulted in complete absence of color development indicating that the chromogenic substance being determined is glycogen uncontaminated with chondroitin sulfate.

The growth hormone used was prepared according to the procedure of Wilhelmi *et al.* (1948) from beef anterior pituitaries and was electrophoretically homogeneous at pH 4.0 and 10.0. The minimum effective doses for the demonstration of contaminating hormones were determined histologically on 26 day old hypophysectomized female rats (injected for 4 days) and were found to be as follows: thyrotropic, 0.5 mg.; interstitial cell stimulating, 0.5 mg.; adrenocorticotropic, >2 mg.; follicle stimulating, >2 mg. The minimum effective dose for growth hormone was 10 gamma as determined by the tibia assay method of Greenspan *et al.* (1949).

RESULTS

Variations in Tibia Glycogen Content with Age in Normal Animals and with Post-operative Period in Hypophysectomized Animals. The tibias of groups of normal female rats varying in age from 12 to 50 days were analyzed for glycogen. The analytical values are presented in Figure 1 in graphical form and are plotted as a function of age. It is evident from the curve that a maximum glycogen content in the tibia is reached at approximately 20 days of age following which there occurs a rapid decline particularly notable between 20 and 26 days and declining less precipitously thereafter. In the period between about 26 and 45 days the glycogen content of the tibia remains quite constant but appears to drop sharply after this age.

Female rats after hypophysectomy at 26 days of age contain considerably less glycogen in their tibias compared to normal animals of corresponding age. The quantitative values are presented in the curve for hypoph-

ysectomized animals in Figure 1. Quite outstanding is the marked decrease in glycogen content of the tibia which occurs during the two weeks following hypophysectomy. Thereafter the decline ceases and a constant glycogen content appears to be maintained.

Effect of Extended Treatment with Growth Hormone on Glycogen Content of Tibia of Hypophysectomized Rats. The animals constituting this series of

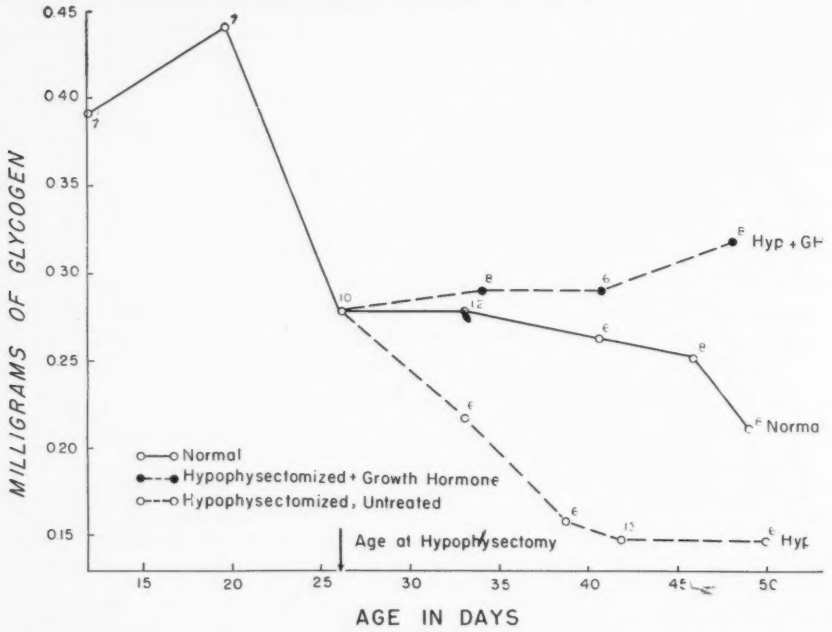

FIG. 1. The variation of tibia glycogen content with age in normal rats, with postoperative period in hypophysectomized rats, and the effect of growth hormone treatment of hypophysectomized rats. The figure adjacent to each point represents the number of tibias analyzed.

experiments were injected with 150 gamma of the purified growth hormone daily beginning with the day following hypophysectomy. One group of animals was thus injected for 7 days, a second for 14, and a third group for 21 days. The results of the various periods of growth hormone treatment are given in Figure 1 (Hyp+G.H.). It is evident that treatment of hypophysectomized female rats under the given conditions maintains the glycogen content of the tibia at a normal level during 1 and 2 weeks of injection with growth hormone following hypophysectomy. Furthermore, whereas the glycogen begins to fall rather sharply after 45 days of age in normal animals, the glycogen content of the treated hypophysectomized

rat tibia of the corresponding age is still increasing after 3 weeks of injection.

Effect of Growth Hormone on Tibia Glycogen Content of Two-week Postoperative Animals. The data presented in the preceding paragraphs indicates that growth hormone is capable of preventing the marked decline in the tibia glycogen content which follows hypophysectomy. In order to determine whether growth hormone is capable of restoring to normal the glycogen content of tibia which are already depleted of glycogen, the following experiment was performed. Hypophysectomized female rats 39 days old, 13 days postoperative were injected daily for 4 days with various doses of growth hormone. The animals were autopsied on the fifth day and tibia glycogen analyzed. The results are presented in Table 1 and indicate that the glycogen content can be restored to normal if sufficiently large doses are given.

TABLE 1. THE EFFECT OF GROWTH HORMONE ON TIBIA GLYCOGEN OF HYPOPHYSECTOMIZED RATS

Number of animals	Type and age	Total dose growth hormone per 4 days (mg.)	Tibia glycogen (mg.)[1]
6	Normal—41 days old	0	0.26 (0.23–0.29)
6	Hypophysectomized 26 day +13 day postoperative	0	0.15 (0.13–0.16)
5	Hypophysectomized 26 day +13 day postoperative	0.1	0.17 (0.16–0.19)
6	Hypophysectomized 26 day +13 day postoperative	0.5	0.20 (0.19–0.23)
6	Hypophysectomized 26 day +13 day postoperative	1.0	0.24 (0.20–0.27)

[1] Mean value and range of variation.

Normal female rats, 50 days old and averaging 110 grams weight, when treated with a 2 mg. dose of growth hormone for 4 days showed no change in tibial glycogen compared with uninjected controls. Over this period of treatment, the injected animals averaged a 5 gram weight gain above the controls.

Effect of Growth Hormone on Tibia Glycogen Content of Fasted Hypophysectomized Rats. In fasting hypophysectomized rats, glycogen stores in the gastrocnemius and heart muscle are abnormally depleted (Illingworth and Russell, 1951). However, when growth hormone is administered to such animals during the fast, restoration of glycogen stores to fasting levels of the intact animal occurs. To determine whether tibia glycogen behaves similarly the following experiment was carried out. Hypophysectomized female rats were fasted 24 hours receiving growth hormone at 0 and 12 hours of fasting. The glycogen content of the tibias as compared to that at onset of fasting and in uninjected fasting hypophysectomized controls at time of autopsy are presented in Table 2. The data indicate

that neither fasting nor growth hormone treatment effected any alteration in the glycogen content of the tibia. The ineffectiveness of growth hormone under these conditions is probably attributable to the relatively short duration of treatment. However, the 24 hour period of fasting is adequate to deplete muscle glycogen stores abnormally in hypophysectomized rats (Russell, 1950).

TABLE 2. THE LACK OF EFFECT OF GROWTH HORMONE ON TIBIA GLYCOGEN OF FASTING HYPOPHYSECTOMIZED RATS

Number of animals	Type and age	Treatment	Tibia glycogen[1] (mg.)
6	11 day postoperative, 39 days old	Onset of fast	0.16 (0.13–0.18)
8	11 day postoperative, 39 days old	Fasted, 0.5 mg. G. H. at 0 and 12 hours	0.15 (0.11–0.19)
7	11 day postoperative, 39 days old	Fasted, uninjected	0.15 (0.12–0.18)

[1] Mean value and range of variation.

SUMMARY

The glycogen content of the tibia from young normal, hypophysectomized, and growth hormone-treated hypophysectomized rats has been presented. The glycogen content of the tibia from immature hypophysectomized rats was found to decrease after operation more markedly than in unoperated animals of corresponding age, and reached a constant low level about two weeks postoperative. By treating hypophysectomized rats with growth hormone daily for one to three weeks after operation, it was possible to maintain the glycogen content of the tibia at normal or higher levels. The administration of growth hormone to hypophysectomized rats already possessing a low content of tibia glycogen increased this to normal when given in sufficiently large doses. Twenty-four hours of fasting did not further alter the low glycogen content of the tibia in hypophysectomized rats, nor was there any detectable change when growth hormone was administered during the fast.

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THE ENTEROHEPATIC CIRCULATION OF THYROXINE IN THE RAT[1]

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IT WAS reported previously that, in the rat, thyroxine is excreted primarily by way of the bile and that the excretion product is a thyroxine-containing compound which we have designated "Compound U" (Taurog, Briggs, and Chaikoff, 1951, 1952). This compound is the normal, physiological excretion product of thyroxine in the rat, and has tentatively been identified as the glucuronide of thyroxine.

The existence of such a mechanism for the excretion of thyroxine raises the question as to its effect on the *reabsorption* of the hormone from the gut. In our earlier work, it was found that a large fraction of injected thyroxine appeared in the feces. This finding, together with the observation that there is no discernible Compound U in rat plasma, led us to suspect that thyroxine, in the form of Compound U, is not well absorbed from the intestinal tract.

The present report describes some experiments designed to study more directly the enterohepatic circulation of thyroxine and the manner in which it is affected by the conversion of thyroxine to Compound U.

EXPERIMENTAL

The general design of the experiments was as follows: Bile was collected by fistula from 2 groups of *donor* rats: 1) rats injected with 100 to 125 μg. of I^{131}-labeled L-thyroxine (rats A–E, Table 1); 2) untreated rats (rats F–L, Table 1). The bile obtained from the untreated rats was made to contain known amounts of either I^{131}-labeled L-thyroxine or I^{131}-labeled Compound U by the addition of purified samples of these compounds (Table 1). The bile samples prepared as described above were then dripped slowly, via the bile duct, into the duodenums of *recipient* rats. (The treatment of both donor and recipient rats is shown in Table 1.) The bile produced by the recipient rats was collected by fistula, analyzed for total I^{131}, and, where the amount of activity was sufficient, fractionated by the method of filter paper chromatography. The details of these procedures are

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TABLE 1. TREATMENT OF DONOR AND RECIPIENT RATS

Recipient rat			Donor rats from which bile was obtained	Treatment of donor rats	I^{131}-compounds in infused bile
No.	Volume of bile infused	Interval during which bile infused			
	cc.	hr.			
1	8	6	A–C (pooled bile)	Injected with 100 μg. radiothyroxine	Compound U (62%); thyroxine (36%)
2	8	6	A–C (pooled bile)	Injected with 100 μg. radiothyroxine	Compound U (62%); thyroxine (36%)
3	8	6	A–C (pooled bile)	Injected with 100 μg. radiothyroxine	Compound U (62%); thyroxine (36%)
4	10	6.5	D	Injected with 125 μg. radiothyroxine	Compound U; thyroxine*
5	10	3	E	Injected with 100 μg. radiothyroxine	Compound U; thyroxine*
6	10	7	F	None	Thyroxine (added)
7	10	7	F	None	Thyroxine (added)
8	11	6	G	None	Thyroxine (added)
9	8	4	H	None	Thyroxine (added)
10	20	9	J	None	Compound U (added)
11	13	6	K	None	Compound U (added)
12	10	7.5	L	None	Compound U (added)

* The bile which was infused into rats 4 and 5 contained much more Compound U than thyroxine, as shown by the radio-autograms of the chromatograms. Quantitative estimates of the relative amounts were not made, however.

described below. Intestinal absorption of the infused I^{131} was determined in the recipient rats by the difference between administered I^{131} and I^{131} appearing in feces and gastrointestinal contents.

Preparation of I^{131}-Labeled L-Thyroxine.—L-thyroxine (Glaxo Laboratories)[3] was incubated at 60° with I^{131}-labeled molecular iodine (I_2^{131}) in 50 per cent ethanol solution at pH 4–5. After 30–60 minutes the thyroxine was precipitated by the addition of water, centrifuged, and washed several times with water. The thyroxine crystals were dissolved in a small amount of 0.02N NaOH, and the desired volume made with isotonic $NaHCO_3$. The specific activity of the final product was generally about 2 μc. per μg. of a purity previously described (Taurog, Briggs, and Chaikoff, 1952).

I^{131}-activity in the various tissues of the rat was determined by dissolving the tissue in strong alkali, drying a small aliquot in a procelain dish, and counting the β-radiation with an end-window Geiger tube. Because of the limitations of this procedure, measurements of I^{131} in the rat carcasses were subject to considerable error, and in the cases where the amount of I^{131} administered to the recipient rats was relatively small, no measurements of carcass-I^{131} are reported.

[3] We are indebted to Dr. B. A. Hems, Research Division, Glaxo Laboratories, Ltd., for a generous supply of L-thyroxine.

RESULTS

I. Experiments with Bile Obtained from Donor Rats Injected with I^{131}-Labeled Thyroxine

Three donor rats (A–C, Table 1) were injected subcutaneously with approximately 100 μg. of I^{131}-labeled L-thyroxine, and their bile ducts were cannulated for bile collection as described elsewhere (Taurog, Briggs, and Chaikoff, 1951). The bile samples obtained during the interval 3–12 hours after the injection were pooled and saved for injection into recipient rats. Analysis of this bile by the method of filter paper chromatography (Taurog, Tong, and Chaikoff, 1950) showed that Compound U accounted for 62 per cent, and thyroxine for 36% of the total radioactivity (Fig. 1a).

To study the enterohepatic circulation of these compounds, 3 recipient rats were treated as follows: Two cannulae were inserted into the bile duct: one was placed in the proximal, or hepatic, end of the duct, and the other,

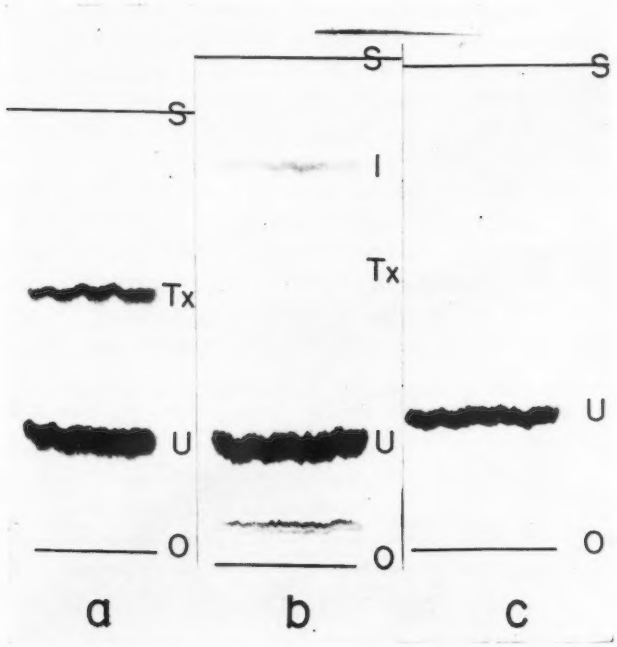

FIG. 1. Radioautograms of ascending filter paper chromatograms: a) Bile from donor rats, A–C, used for infusion into recipient rats, 1–3; b) Bile collected from recipient rat 7, 7.5–18.5 hours after infusion of bile containing added I^{131}-thyroxine; c) Bile containing added Compound U for infusion into recipient rat 10, prepared as described in text. Solvent, collidine-water (100:35) in an ammonia atmosphere. O, origin; U, Compound U; Tx, thyroxine; I, inorganic iodide; S, solvent front.

in the distal, or duodenal, end (Siperstein, Hernandez, and Chaikoff, in press). The cannula in the distal segment was attached to the barrel of a 10-cc. syringe through a drip apparatus. 8 cc. of the bile obtained from the donor rats referred to above were added to each of the 10-cc syringes. The height at which the syringe was held served to control the rate at which the bile dripped into the rat, and this was made to approximate the rate at which the recipient rat produced bile. Each recipient rat received bile for a period of 6 hours, whereas the bile was collected from this same rat for 65 hours. During that 65 hours, bile was collected serially in order to determine the rate as well as the extent of the recirculation of I^{131}.

The rate of appearance of the I^{131} in bile of recipient rats 1–3 is shown in Figure 2A. Over the entire period of 65 hours, only 5–15% of the administered I^{131} appeared in bile. The greatest part of this was secreted during the first 15 hours. In 2 other recipient rats (4 and 5, Fig. 2A) infused with bile from separate donor rats (D and E, Table 1), 9.3 and 7.6% of the administered I^{131} appeared in the bile in 41 and 51 hours, respectively. Thus it would appear that after an injection of I^{131}-labeled thyroxine only about 10% of the I^{131} excreted into the bile undergoes enterohepatic circulation.

The fate of the rest of the I^{131} that recipient rats 1–3 had received is shown in Table 2. By far the largest fraction of the I^{131} infused into the duodenums of the recipient rats was found in the feces (64–76%). It is unlikely, on the basis of previous work by Gross and Leblond (1947), that any appreciable part of the fecal I^{131} represents iodine which had been absorbed and subsequently resecreted through the intestinal wall. The large fraction of the I^{131} appearing in the feces, therefore, makes it evident that approximately 70% of the infused I^{131} was not absorbed. Of the 30% that was absorbed, only about one third was recirculated into bile.

TABLE 2. FATE OF ENTERALLY ADMINISTERED I^{131} IN RECIPIENT RATS
(The rats recorded below are the same as those referred to in Figure 2. All values refer to percentages of the administered I^{131})

Recovery	Infused with bile obtained from I^{131}-thyroxine-injected donor rats			Infused with bile containing added I^{131}-thyroxine				Infused with bile containing added I^{131}-Compound U		
	Rat 1 65 hrs.	Rat 2 65 hrs.	Rat 3 65 hrs.	Rat 6 64 hrs.	Rat 7 64 hrs.	Rat 8 37½ hrs.	Rat 9 67 hrs.	Rat 10 68½ hrs.	Rat 11 67½ hrs.	Rat 12 49 hrs.
	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}	Per cent of administered I^{131}
Bile	10.9	14.7	5.5	20.6	19.5	17.1	31.0	20.3	9.6	12.2
Feces + gastrointestinal contents	64.6	64.0	75.8	58.8	43.4	44.0	31.8	67.5	75.8	60.0
Urine*	2.0	0.8	2.2	3.0	2.5	4.6	3.6	0.3	0.9	0.7
Gut	0.5	0.7	0.1	0.3	0.5	0.7	2.9	†	1.8	2.1
Thyroid	†	†	†	3.1	3.7	3.8	6.8	4.7	2.6	2.7
Carcass	11.7	18.3	4.6	11.5	15.4	‡	‡	‡	‡	‡

* The urine values are only approximate since the urine samples were contaminated with feces.
† Measurement not made.
‡ Concentration of I^{131} too low for measurement.

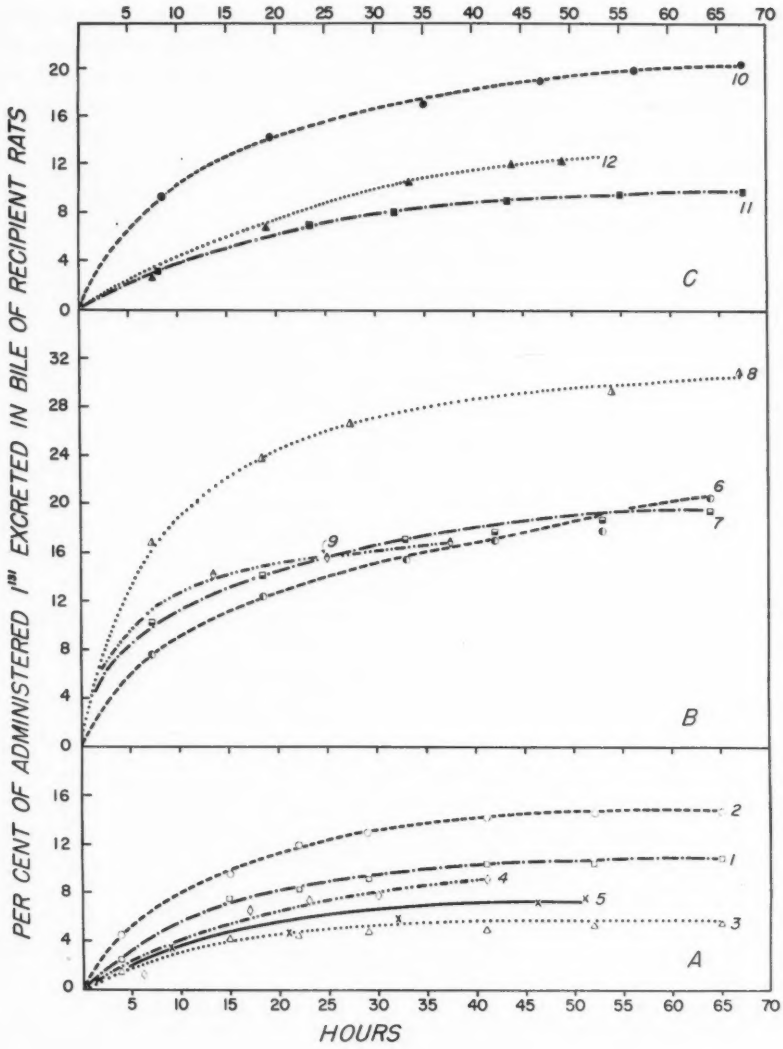

FIG. 2. Cumulative excretion of I^{131} in bile of recipient rats: A) Rats 1–5, infused with bile obtained from I^{131}-thyroxine injected rats; B) rats 6–9, infused with bile containing added I^{131}-thyroxine; C) rats 10–12, infused with bile containing added I^{131}-Compound U.

II. Experiments with Bile to Which I^{131}-Labeled L-Thyroxine Was Added

The experiments described above were performed with bile containing both thyroxine and Compound U. It became of interest to determine whether the enterohepatic circulation of these 2 compounds differed, and the experiments designed to test this point are described in the present and following sections.

Nonradioactive bile was collected from donor rats that had *not* been injected with I^{131} (F–H, Table 1). To this bile was added I^{131}-labeled L-thyroxine, prepared as described above. 8–11 cc. of such thyroxine-containing bile were then infused slowly into the duodenums of 4 recipient rats via the bile duct, just as described in Section I. Rats 6 and 7 received, in this manner, approximately 8 μg. of thyroxine iodine, whereas rats 8 and 9 were infused with approximately 0.9 and 0.5 μg. of thyroxine iodine, respectively. Bile was collected from rats 6 and 7 for 64 hours, and from rat 8 for 67 hours. Rat 9 removed its bile cannula after 37.5 hours, and the bile collection was therefore terminated at that interval.

The rate of appearance of the I^{131} in the bile of the recipient rats is shown in Figure 2B. 17–31% of the infused I^{131} appeared in bile during the collection periods. These values are significantly higher than the corresponding values observed for rats 1 to 5 ($p < 0.01$). The biliary excretion of the administered I^{131} by rats 6 and 7, which received 8 μg. of thyroxine iodine, did not differ appreciably from that by rats 8 and 9 which received one-tenth as much thyroxine.

The bile collected from rats 6 and 7 was analyzed by the method of filter paper chromatography. The radioautograms (Fig. 1b) show that the thyroxine which recirculated into the bile was present primarily in the form of Compound U. Only a very small part of the I^{131} appearing in bile was unchanged thyroxine. The bile from rats 8 and 9 did not contain a sufficient concentration of I^{131} to permit analysis by paper chromatography.

The fate of the remainder of the thyroxine I^{131} infused into recipient rats 6 to 9 is shown in Table 2. The largest fraction of the I^{131} was again found in the feces. However, the total absorption of I^{131} was 41–68%, significantly higher ($p \sim .02$) than the absorption of I^{131} in rats 1–3 (24–36%, Table 2).

III. Experiments with Bile to Which I^{131}-Labeled Compound U Was Added

Purified samples of Compound U were obtained in the following manner: A rat was injected subcutaneously with 100–150 μg. of I^{131}-labeled L-thyroxine, and its bile duct was cannulated. The bile was collected during an interval when the concentration of Compound U was maximum (e.g., 8 to 14 hours). It was brought to pH 3.5–4.0 with dilute HCl, and extracted with an equal volume of n-butanol. A total of 200 μl. of the butanol extract

was delivered in 25 μl. portions on each of 24 filter paper strips. Compound U was separated from other I^{131}-containing components of bile by chromatography in collidine-water-NH_3, as previously described (Taurog, Briggs, and Chaikoff, 1952). The sections of the filter paper chromatograms corresponding to Compound U were cut out, and each was eluted with approximately 0.3 ml. of .02N NaOH. The eluates of the 24 strips were combined and concentrated to a volume of approximately 1 ml. *in vacuo* at about 45°.

Three samples of Compound U were prepared as described above. The quantity of Compound U iodine was estimated from its radioactivity and from the known specific activity of the injected thyroxine iodine, on the assumption that the specific activity of the Compound U iodine was very close to that of the injected thyroxine iodine. The Compound U samples were added to nonradioactive bile obtained from donor rats J–L, for infusion into recipient rats 10–12. Rat 10 received approximately 0.5 μg. of Compound U iodine in 20 cc. of bile; rat 11 received approximately 1.5 μg. of Compound U iodine in 10 cc. bile; and rat 12, approximately 2 μg. of Compound U iodine in 13 cc. bile. The infused bile samples were analyzed by paper chromatography. At least 90% of the I^{131} was present in the form of Compound U (Fig. 1c).

The rate at which the infused Compound U-I^{131} appeared in the bile of the recipient rats is shown in Figure 2C (rats 10–12). Values of 9.6, 12.2, and 20.3% of the administered I^{131} were obtained in 49 to 68 hours. These values appear to be somewhat lower than those obtained after the infusion of bile containing I^{131}-thyroxine (rats 6 to 9, Fig. 2B), although the difference between the mean values is not statistically significant ($0.2>p>0.1$).

The fate of the remainder of the I^{131} in rats 10–12 is shown in Table 2. By far the largest fraction of the administered I^{131} appeared in the feces 60–76%). The absorption of I^{131} in these rats (24–40%) was significantly less ($p\sim0.02$) than the absorption of I^{131} in the rats which received labeled thyroxine (41–68%). It may be concluded, therefore, that Compound U is less readily absorbed than thyroxine from the gastrointestinal tract of the rat.

DISCUSSION

It has been demonstrated by many investigators, using rabbits (Elmer and Luczynski, 1933), cats (Clayton *et al.*, 1950), and dogs (Asimow and Estrin, 1931; Schittenhelm and Eisler, 1932), that a significant portion of an enterally administered dose of thyroxine iodine appears in the bile. The nature of this iodine could not be determined with certainty by previous methods, but several workers have suggested the possibility of an enterohepatic circulation of thyroxine (Leblond, 1951; Albert *et al.*, 1952).

The method of filter paper chromatography has now made it possible to

separate thyroxine from other closely related iodine compounds. The application of this method of analysis to bile collected from rats injected with small doses of I^{131}-labeled thyroxine has revealed that, although some unchanged thyroxine is present, most of the I^{131} is in the form of a conjugated thyroxine compound which we have called Compound U.

In the experiments reported here, we have attempted to measure the intestinal absorption and the enterohepatic circulation of thyroxine and of its excretion product, Compound U. Bile obtained from rats injected with 100 to 125 μg. I^{131}-thyroxine, and containing I^{131} in the form of both Compound U (approximately $\frac{2}{3}$) and thyroxine (approximately $\frac{1}{3}$), was slowly infused, via the bile duct, into the duodenums of recipient rats. Approximately 30% of the I^{131} was absorbed. The bile secreted by the recipient rats contained 5.5 to 14.7 (average 9.6%) of the administered I^{131}, or approximately $\frac{1}{3}$ of the absorbed I^{131}. From these data it may be concluded that of the iodine which is secreted into the bile of a rat following the injection of thyroxine, only about 10% undergoes enterohepatic circulation.

Experiments were carried out to determine whether there was any appreciable difference between unchanged thyroxine and Compound U in the extent of their absorption and enterohepatic circulation. In these experiments, purified samples of I^{131}-thyroxine or of I^{131}-Compound U were added to nonradioactive bile. The bile prepared in this manner was then infused into the duodenums of recipient rats from which bile was also collected. The rats that received bile containing added I^{131}-thyroxine absorbed 41–68 (average 58) % of the administered I^{131}, significantly more than the rats that received bile containing I^{131}-Compound U. In both groups, $\frac{1}{3}$ to $\frac{1}{2}$ of the absorbed I^{131} was recirculated into the bile, mostly in the form of Compound U.

It does not seem likely that absorption of Compound U depends on its hydrolysis to thyroxine. It was reported previously (Taurog, Briggs, and Chaikoff, 1952) that Compound U is not hydrolyzed by the enzymes of the upper intestinal tract, although it is hydrolyzed by the contents of the lower bowel.

The finding that Compound U is less readily absorbed from the gastrointestinal tract than is thyroxine provides added evidence that the conversion of thyroxine to Compound U is a mechanism for ridding the organism of excess thyroxine more efficiently. It is pointed out elsewhere (Briggs, Brauer, Taurog, and Chaikoff, in press) that the liver secretes Compound U into the bile more readily than it does unchanged thyroxine, and that the conversion of thyroxine to Compound U in the rat is, therefore, probably a simple detoxification reaction. This detoxification mechanism, however, is quite limited in the rat, and seems to break down when very large doses of thyroxine are administered.

In a recent paper, Albert *et al.* (1952) reported on the absorption of thyroxine from isolated portions of the gastrointestinal tract of the rat. Using I^{131}-labeled L-thyroxine obtained from Abbott Laboratories, these workers reported 20 to 25% absorption from the small bowel. These values are somewhat lower than the ones observed here.

SUMMARY

1. The absorption and enterohepatic circulation of thyroxine and its excretion product, Compound U, were studied in the rat.

2. Bile obtained from rats injected with 100 to 125 μg. of I^{131}-labeled L-thyroxine was infused slowly, via the bile duct, into the duodenums of recipient rats. The I^{131} in the infused bile, which was largely in the form of Compound U, was absorbed to the extent of 25 to 35%. 5.5 to 14.7% of the administered I^{131} appeared in the bile secreted by the recipient animals in 65 hours.

3. I^{131}-labeled L-thyroxine was added to nonradioactive bile and infused slowly, via the bile duct, into the duodenums of recipient rats. The absorption of the thyroxine iodine varied from 41 to 68%. The I^{131} which appeared in the bile secreted by the recipient rats varied from 17 to 31% of the administered I^{131}.

4. I^{131}-labeled Compound U, isolated by elution from filter paper chromatograms, was added to nonradioactive bile and infused slowly into the duodenums of recipient rats. The absorption of the iodine varied from 24 to 40%. The I^{131} which appeared in the bile of the recipient rats varied from 9.6 to 20.3%.

5. Compound U is less readily absorbed from the gastrointestinal tract than is thyroxine. The conversion of thyroxine to Compound U in the rat therefore permits the animal to eliminate excess thyroxine somewhat more efficiently.

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THE EFFECTS OF IODIDE, PERCHLORATE, THIOCYANATE, AND NITRATE ADMINISTRATION UPON THE IODIDE CONCENTRATING MECHANISM OF THE RAT THYROID*

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THE thyroid gland of an animal chronically treated with a thiouracil-like drug, although unable to manufacture thyroid hormone, manifests a remarkable ability to collect and concentrate the iodide ion (Vanderlaan and Vanderlaan, 1947; Taurog, Chaikoff, and Feller, 1947). About one hour after the subcutaneous administration of iodide in rats the thyroid exhibits an average concentration gradient of 250 over the serum. Taurog, Chaikoff, and Feller (1947) noted this ratio to be unchanged when 2 μg. or 100 μg. of iodide were given to adult rats. Vanderlaan and Vanderlaan (1947) found that the gradient remained constant when doses up to 100 μg. of potassium iodide were administered in young rats, but that when larger quantities were given it diminished progressively. However, even when 10,000 μg. were given the gradient was not abolished completely, a concentration of 3 fold persisting in favor of the gland.

In contrast to the action of the thiouracil-like agents, the antithyroid action of thiocyanate is characterized by interference with thyroidal collection of inorganic iodide (Franklin, Chaikoff, and Lerner, 1944). Thiocyanate has also been shown to discharge iodide previously accumulated by the thyroid gland of the rat treated with propylthiouracil (Vanderlaan and Vanderlaan, 1947). Recently, it has been found (Wyngaarden, Wright, and Ways, 1952) that certain monovalent anions, principally the halogen oxides, share with thiocyanate the properties of inhibiting collection and of interfering with retention of the iodide ion within the thyroid gland. Perchlorate, the most potent anion of this group, was 10 times as effective as thiocyanate in this capacity. The effectiveness of perchlorate as an

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iodide discharging agent and as an uptake inhibitor has also been demonstrated in the thyroid patient (Stanbury and Wyngaarden, 1952).

The present report describes studies upon the comparative effects of graded "carrier" doses of four agents, perchlorate, thiocyanate, inert iodide, and nitrate, upon the ability of the thyroid gland to maintain a concentration gradient of radioiodide over the serum.

METHODS

Male Wistar rats in groups of 24–36 animals, having group average weights of 155–196 gm., were treated for 14 or more days with the antithyroid regimens specified in Table 1. In most experiments, one hour prior to the administration of radioiodide the rats received 10 mg. of propylthiouracil intraperitoneally to supplement the dietary antithyroid agent. Ten μc. of radioiodide were then administered subcutaneously in 1 cc. of water, which also contained the desired quantity of the "carrier" anion. Potassium iodide as carrier was given in doses up to 10,000 μg. per rat. The other compounds were administered in molar equivalents of specific dose levels of potassium iodide. One hour following the administration of radioiodide the rats were killed with sodium pentobarbital administered intraperitoneally. Blood was withdrawn from the inferior vena cava and the serum separated. Thyroid glands were removed, weighed on a Roller-Smith balance, and digested in 3 cc. of 5% potassium hydroxide containing 10 μg. of sodium iodide. Two-tenths cc. of serum or of thyroid digest were then evenly distributed upon silvered copper planchets, and their radioactivity was determined with the Geiger-Muller counter. The ratio of radioactivity between thyroid and serum was determined for each rat, from the activity data per gram of thyroid and per cc. of serum (without correcting for specific gravity). No correction was applied for the variations of average weight from group to group.

RESULTS

In Table 1 are given the individual thyroid-serum ratios of radioiodine and the gradient averages at each dose level for each group. The minimal dose levels effecting statistically significant reductions of the average collection gradients, expressed in micrograms of potassium iodide, or in equivalent doses of other agents, are: KI, 1000; KSCN, 1000; KNO_3, 1000; $KClO_4$, 30.

The average gradients have also been expressed as percentages of the average control gradient. From these values composite averages, adjusted in each computation for the size of the series, have been calculated for each compound, and these results are plotted in Figure 1. It can be seen that perchlorate was distinctly more potent than the other ions. Not only was a significant reduction of the gradient produced by a relatively small dose of this substance, but in the rats receiving potassium perchlorate in doses equivalent to 10,000 μg. of potassium iodide, the radioiodide accumulation gradient was virtually abolished.

The approximately equal effectiveness of molar equivalent doses of iodide and of thiocyanate is to be noted. This result had been anticipated

TABLE 1. RATIO OF THYROID RADIOACTIVE IODIDE TO SERUM RADIOACTIVE IODIDE IN RATS INJECTED WITH INCREASING DOSES OF POTASSIUM IODIDE AS CARRIER, OR OF EQUIVALENT DOSES OF THE OTHER AGENTS, ACCOMPANYING A CONSTANT TRACER DOSE OF RADIOACTIVE IODIDE

Compound	Group	Pretreatment*	Micrograms of potassium iodide, or equivalent dose of other agent											
			0	3	10	30	100	300	1,000	3,000	10,000	30,000	100,000	300,000
KI	I	Reg. TU	93				136	112	51		8			
			66				59	78	66		8			
			63				50	42	55		11			
			50				139	102	91		6			
			45				79	68	46		9			
							115	71	40		11			
		Av.	63 ±5.6‡ (100%)				96 ±10.7 (152%)	79 ±6.8 (125%)	58 ±5.0 (92%)		9±.5 (14%)			
	II	Reg. TU	61			73		50	43	13	9			
			96			121		64	32	24	10			
			87			92		39	67	17	11			
			42			38		78	26	25	8			
			36			90		75	41	15				
		Av.	64±8.0			83±9.2 (130%)		61 ±5.0 (95%)	42 ±4.7 (66%)	19 ±1.6 (30%)	10±.5 (16%)			
	III	Reg. TU†	124		65		93	80	79	19	9			
			98		166		138	77	89	28	12			
			83		118		112	84	54	20	12			
			136		12		78	113	97	22	16			
								127	100	49				
		Av.	110±8.1		90±22.4 (82%)		108 ±7.8 (98%)	96 ±6.8 (87%)	84 ±5.6 (76%)	28 ±4.9 (25%)	12±1.4 (11%)			
Total Av.			100%		82%	130%	129%	104%	79%	28%	14%			
KSCN	I	Reg. TU†	250			377		412	327	26	37			
			242			447		301	108	42	102			
			419			135			83	141	19			
						348			130					
		Av.	330±40.9			327±45.3 (99%)		356 (108%)	162± ±37.6 (49%)	70 ±24.1 (21%)	56±17.1 (17%)			
	II	Rem. TU†	206			79		168	42	29	14			
			131			131		76	36	11	6			
			61			40		40	54	23	22			
			43			51		35	77	23	5			
			85			89		83	57	12	3			
			113			135		40	32	19				
		Av.	107±16.1			88±10.9 (82%)		74 ±13.9 (69%)	50 ±4.6 (47%)	20 ±1.9 (19%)	10±2.4 (9%)			
	III	Rem. TU†	152			204		131	76	42	4			
			201			254		77	82	36	6			
			213			71		30	180	67	5			
			91			359		47	54	41	4			
			216			96		254	86	10	12			
									222	94				
		Av.	175±16.1			197±35.5 (113%)		108 ±27.3 (62%)	115 ±18.6 (66%)	48 ±7.9 (27%)	6±1.0 (3%)			
Total Av. %			(100%)			(97%)		(72%)	(55%)	(23%)	(6%)			
$KClO_4$	I	Rem. PTU	214			145	56	15	5	3	2			
			148			102	115	27	8	3	2			
			265			59	72	17	7	5	2			
			181			106	41	30	7	5	1			
			119			69	31	19	8	4				
		Av.	185±17.2			96 ±10.3 (52%)	63 ±9.8 (34%)	22 ±2.0 (12%)	7 ±0.4 (4%)	4 ±0.3 (2%)	2±0.2 (1%)			

TABLE 1—*Continued*

Compound	Group	Pretreatment*	Micrograms of potassium iodide, or equivalent dose of other agent											
			0	3	10	30	100	300	1,000	3,000	10,000	30,000	100,000	300,000
$KClO_4$	II	Rem. PTU	391	201	209	104	82		8					
			124	176	209	185	82		12					
			358	138	176	155	57		13					
			271	136	300	168	27		13					
				429	144	61	67		11					
		Av.	264 ±33.4	215 ±36.8 (82%)	208 ±17.6 (79%)	135 ±15.4 (51%)	63±9.9 (24%)		11±0.6 (4%)					
		Total Av. %	(100%)	(82%)	(79%)	(52%)	(29%)	(12%)	(4%)	(2%)	(1%)			
KNO_3	I	Rem. PTU	207			313		203	192	180	245			
			168			297		74	142	136	116			
			256			62		194	29	86	42			
			238			89		72	28	63	193			
			81			142		69	112	56	39			
		Av.	190±21.0			181±35.4 (95%)		122 ±21.0 (64%)	101 ±21.6 (53%)	104 ±15.9 (55%)	127 ±27.5 (67%)			
	II	Rem. PTU	521				218		394		291	110	36	25
			251				456		159		157	248	42	18
			209				222		161		261	135	31	17
			398				176		112		132	120	17	31
							131		105		191	47	39	7
		Av.	345±48.0				241±30.9 (70%)		186±36.0 (54%)		206 ±20.8 (60%)	132 ±22.0 (38%)	33 ±3.0 (10%)	20 ±2.7 (6%)
		Total Av. %	(100%)			(95%)	(70%)	(64%)	(54%)	(55%)	(64%)	(38%)	(10%)	(6%)

* Remington diet (*J. Nutrition*, 13: 223, 1937).
Regular diet (Purina Fox Chow. Iodine=0.32 mg./100 gm.).
Thiouracil, 0.2% drinking water.
Propylthiouracil, 0.1% in diet.
† Priming dose of 10 mg. propylthiouracil not given.

‡ Averages represent: Mean± probable error of the mean (R). $R = .6745\sqrt{\frac{\Sigma(V^2)}{n(n-1)}}$.

(Stanbury, 1951) from a comparison of the data of Vanderlaan and Vanderlaan (1947) concerning the effects of carrier iodide upon the concentrating mechanism, with those of Raben and Astwood (1949) concerning the effects of thiocyanate.

It is apparent that considerably larger equivalent doses of potassium nitrate are required to reduce the gradient to 10% or less of the control level than of the other compounds, despite the fact that in terms of minimal effective dose, nitrate is similar to iodide and thiocyanate. It is noteworthy (Table 1) that some rats appear to be quite resistant to potassium nitrate even in doses of 6–18 mg., the equivalents of 10,000 to 30,000 μg. of potassium iodide.

DISCUSSION

These results are quantitatively in general agreement with those concerning the effectiveness of perchlorate, thiocyanate and nitrates as dischargers of thyroidal iodide in the intact rat (Wyngaarden, Wright, and Ways, 1952). In the present study, the order of effectiveness of these agents

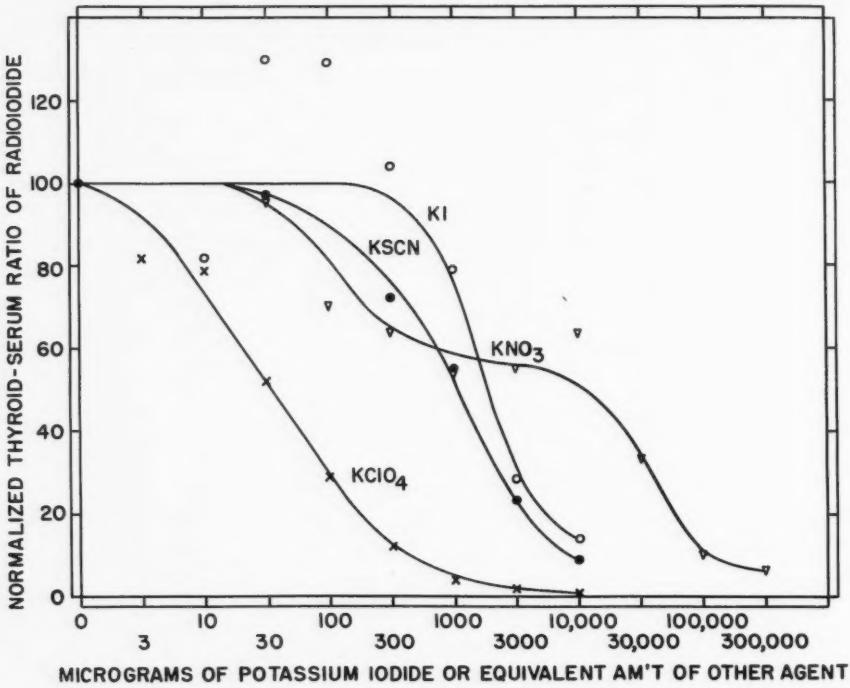

FIG. 1. The effects of iodide, thiocyanate, perchlorate, and nitrate ions upon the concentration gradient of I^{131}, when these agents were given in increasing doses, accompanying a constant dose of I^{131}. The ordinate is thyroid-serum I^{131} ratio, normalized to express the control gradient for each group as 100, and averaged for each agent. The abscissa is the dose of potassium iodide or equivalent dose of thiocyanate, perchlorate, or nitrate, plotted logarithmically.

in producing a profound depression of the accumulation gradient is: perchlorate, thiocyanate, iodide and nitrate. In contrast to perchlorate, thiocyanate and nitrate, which reduce simultaneously the concentration gradient and the total iodide content of the gland due to an inhibition of uptake, iodide exerts its effect upon the gradient by leading to a partial saturation of the collecting mechanism (Vanderlaan and Vanderlaan, 1947).

Since the efficiency of any anion in discharging iodide *in vivo* will necessarily depend in part upon its extra-thyroidal disposal rate, studies of the effects of these anions upon tissue slices under conditions of controlled concentration will be necessary to establish more exactly the relative

potencies of antithyroid action. The present evaluations, obtained in the intact animal, may be subject to revision when such data are available.

The mechanism of action of these agents has not been established. Simple competition for collection by the thyroid cannot account for the action of this anion group, since thyroidal accumulation of thiocyanate ion has been excluded by Wood and Kingsland (1950), and that of another antithyroid anion, iodate, by Leblond and Sue (1941). The preferential inhibition of thyroid function caused by these ions is apparently not accompanied by preferential concentration. Yet the effect of excess iodide in abolishing the antithyroid action of thiocyanate (Astwood, 1943) and of overcoming the interference with iodide collection caused by perchlorate (Wyngaarden, Wright, and Ways, 1952) suggests that a competitive mechanism is involved.

In preliminary studies on the effects of graded carrier doses of potassium iodide, rats were employed which had been made goitrous either by using a fox chow or a Remington low iodine diet to which had been added either thiourea or thiouracil (cf. Table 1). These regimes resulted in comparable degrees of thyroidal enlargement. The mechanism of action of thiouracil and propylthiouracil are considered to be identical. The quantities of iodide consumed daily by the animals on the Remington or the fox chow diet were far below the smallest dose of carrier iodide which resulted in any change in the thyroid-serum iodide ratio. It would appear that the effects of the anions studied were independent of the regimen employed for goiter production.

CONCLUSIONS

The effects of increasing quantities of inert iodide, thiocyanate, perchlorate and nitrate upon the ability of the thyroid gland to concentrate radioiodide have been studied in the rat. All of these agents caused a marked depression of the iodide concentration gradient existing between thyroid and serum, when given in sufficient quantities. The most potent antithyroid anion was perchlorate, the least, nitrate. The effects of molar equivalent doses of inert iodide and of thiocyanate upon this gradient were approximately equal. The mechanism of action of these anions is unknown. It is probable that the apparent qualitative similarity of perchlorate, thiocyanate, and nitrate with that of excess iodide is only superficial.

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THE ADRENAL CORTEX AND SULFUR METABOLISM: THE INFLUENCE OF AMINO ACID SULFUR ON THE FORMATION OF LIVER GLYCOGEN[1]

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A NUMBER of years ago it was demonstrated that oral or percutaneous administration of sulfur to rats or rabbits resulted in adrenal hyperplasia and marked deposition of liver glycogen (Burgi and Gordonoff, 1926; Foldes, 1928). The possible interrelation of these two observations remained obscure at that time, for the glycogenic activity of 11-oxy corticoids was as yet unsuspected. In retrospect, it seems likely that the administered sulfur induced an adrenal hyperplasia and an outpouring of adrenal cortical hormones which resulted in excessive liver glycogen deposition.

Recent studies indicating an intimate association between adrenal cortical function and sulfur metabolism (Goldzieher and Edlin, 1953; Goldzieher, Rawls and Goldzieher, 1953) have suggested further exploration of these findings. In view of the fact that colloidal sulfur, though of a very low order of toxicity, is still a foreign substance and its administration may constitute a nonspecific stress, it was decided first to investigate the effect of sulfur in physiological form. Studies were accordingly carried out with *l*-cysteine, *l*-cystine, glutathione, and the sulfurless analogue of cysteine, serine, in order to determine whether or not the presence of sulfur exercised a specific effect on the ability of these substances to cause deposition of liver glycogen and furthermore, whether or not such effects were influenced by the presence or absence of cortical hormones.

MATERIALS AND METHODS

Adult CFW male rats weighing 200 to 250 gm. were maintained on a stock diet of Purina fox checkers and water *ad lib.* The intact animals were fasted for 12, 24 or 48 hours before administration of the test substance. In other animals, adrenalectomy was performed under nembutal anesthesia; they were then maintained on 10% glucose in saline for one day, on saline and an *ad lib.* diet for one day, and fasted for 24 hours before use. The test substance was administered intraperitoneally or by stomach tube as indicated; controls received isotonic saline intraperitoneally or tap water by stomach

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tube. In acute experiments the animals were killed exactly three hours after administration of the test substance, by a blow on the head. Fragments of approximately one gram weight were obtained from two different areas of the liver, dropped into tared centrifuge tubes containing 30% potassium hydroxide, weighed as rapidly as possible, and placed immediately into the steam bath. The technique of Venning was used for for the isolation of glycogen; its subsequent hydrolysis was carried out by the method of Good, Kramer and Somogyi, and aliquots of the resultant hydrolysate were then run in duplicate by the Nelson and the Folin-Wu methods for reducing substances. Results are expressed as per cent of tissue weight (i.e., grams glycogen as glucose, per 100 grams liver tissue). In other experiments which were intended to test the occurrence of progressive glycogen storage upon repeated administration of cystine, the animals were killed 24 hours after the last dose of cystine. Half of this group was starved for 12 hours, the remainder for 24 hours prior to sacrifice.

Cysteine was neutralized with the calculated amount of sodium hydroxide immediately before use, serine and reduced glutathione were dissolved in saline; cystine was given as a suspension in tap water. The amount of fluid fed or injected was kept at 2 to 3 ml. per animal.

RESULTS

The data are summarized in Table 1.

Intact animals.—One group of intact animals was fasted for 48 hours, and the glycogen concentration in the controls was observed to be 0.698 $\pm$0.229%. No significant increase in liver glycogen followed the injection of serine (liver glycogen 0.857 $\pm$ 0.420%; p = 0.4), the injection of cysteine (0.601 $\pm$ 0.123%) or the oral administration of cysteine (0.624 $\pm$ 0.222%). There was clearly no difference between the glycogenic activities of serine and cysteine under these conditions.

Another group of animals was fasted for 24 hours and the control liver glycogen concentration was found to be 0.419 $\pm$ 0.155%. When similarly

TABLE 1. INTACT RATS

Test substance	No. of animals	Dose mg./100 Gm.	Route	Duration of fast, hours	Liver glycogen concentration (Nelson), per cent $\pm$st. deviation
control (saline)	8	1 ml.	i.p.	48	0.698 $\pm$ 0.229
dl-serine	6	25	i.p.	48	0.857 $\pm$ 0.420
l-cysteine	7	25	i.p.	48	0.601 $\pm$ 0.123
l-cysteine	5	75	oral	48	0.624 $\pm$ 0.222
glutathione	6	25	i.p.	48	0.424 $\pm$ 0.069
control (water)	4	1 ml.	oral	24	0.419 $\pm$ 0.155
l-cysteine	4	75	oral	24	1.084 $\pm$ 0.249
l-cystine $\times$25	4	10	oral	24	0.484 $\pm$ 0.123
control (water)	4	1 ml.	oral	12	1.43 $\pm$ 0.270
l-cystine $\times$25	4	10	oral	12	1.27 $\pm$ 0.401
		ADRENALECTOMIZED RATS			
control (saline)	5	1 ml.	i.p.	24	0.029 $\pm$ 0.004
dl-serine	5	25	i.p.	24	0.063 $\pm$ 0.005
l-cysteine	6	25	i.p.	24	0.041 $\pm$ 0.012
l-cysteine	3	75	oral	24	0.106 $\pm$ 0.020
glutathione	4	25	i.p.	24	0.026 $\pm$ 0.007

fasted animals were given cysteine by mouth in a dose of 75 mg./100 gm. body weight, liver glycogen accumulation was observed, for the concentration rose to $1.094 \pm 0.249\%$. It is interesting that cysteine deposited substantial amounts of glycogen following a 24-hour fast, but not after a 48-hour fast. This may well explain the divergent results obtained by various investigators; Hess (1949), for example, obtained very low liver glycogen concentration after 24 hours' starvation and found a substantial glycogenic effect with cysteine whereas Medes and Vitagna (1939), who starved animals longer, did not get glycogen depletion of this order, and were not able to observe glycogenic activity due to cysteine. No satisfactory explanation for this effect of starvation is at hand. It is of course quite possible that the amino acids still have glycogenic activity, though of a lesser degree, in the 48-hour fasted animal, but the prevailing high concentration of glycogen masks this effect. It is equally possible that the presence of adequate depots of glycogen in the liver is not conducive to additional storage.

Glutathione in the intact, 48-hour fasted animal showed anomalous behavior, for the liver glycogen concentration following injection of this substance was $0.424 \pm 0.069\%$, which is significantly lower ($p = 0.04$) than the control glycogen level of $0.698 \pm 0.229\%$.

The response of rats to repeated administration of cystine was studied in further experiments. An aqueous suspension of cystine was administered daily to 8 animals for 25 days. Half of the group was sacrificed after a 12-hour fast and the remainder after 24 hours' starvation. The liver glycogen concentration of the cystine-treated, 12-hour fasted group, $1.27 \pm 0.40\%$, did not exceed that of the 12-hour fasted, tap-water-treated controls ($1.43 \pm 0.27\%$), nor was there any change after 24 hours' fast, the concentration of liver glycogen of the cystine-treated and control groups being 0.484 ± 0.123 and $0.419 \pm 0.155\%$ respectively.

Adrenalectomized animals.—A 24-hour fast in the operated animals reduced the liver glycogen concentration to $0.029 \pm 0.004\%$. The intraperitoneal administration of serine raised the liver glycogen significantly to $0.063 \pm 0.005\%$ within 3 hours, and a significant though somewhat smaller increase to $0.041 \pm 0.012\%$ occurred after cysteine injection. The intragastric administration of cysteine at 3 times the concentration used previously caused the deposition of somewhat less than 3 times as much liver glycogen ($0.106 \pm 0.020\%$). Under these circumstances, apparently, the sulfhydryl group does not have any specific effect on glycogen accumulation.

It is interesting to note that glutathione under the same conditions does not bring about any deposition of glycogen, for the concentration after such treatment ($0.026 \pm 0.007\%$) does not differ from that of the controls.

DISCUSSION

The data just presented do not reveal any exceptional glycogenic activity ascribable to the various forms of sulfur administered. Hess has studied the effect of some of these compounds in rats with livers almost completely depleted of glycogen, and found the concentration of glycogen after serine administration to be 1.08% after 2 hours and 0.52% after 3 hours; following cysteine administration the values were 0.83 and 1.11% respectively. In other words, the magnitude of the glycogenic response was the same, although the rapidity of accumulation and subsequent disappearance varied. In our studies with partially depleted rats, the findings are analogous, for neither cysteine nor serine added a substantial increment to the prevailing liver glycogen level.

The role of the adrenal cortex in this process was explored in adrenalectomized animals. In the severely depleted livers of this group both serine and cysteine caused glycogen deposition of comparable magnitude, indicating that adrenalectomy did not prevent the conversion of amino acid to liver glycogen, and furthermore, that the sulfur moiety of the compounds tested did not have an action either in the presence or absence of cortical hormones.

The foregoing experiments differed in duration as well as in test substance from those of Foldes (1928) or Burgi and Gordonoff (1928). For this reason, comparably long-term studies using a compound more physiological than colloidal sulfur were initiated by feeding cystine at a dosage level corresponding to 3.1 mg. sulfur per 100 gm. body weight per day for a period of 25 days. Administration of this substance, which is readily converted to cysteine, avoided changes in electrolyte intake or balance which might have occurred if cysteine hydrochloride, either as the acid salt or neutralized with sodium hydroxide, had been used. In this instance also, there was no appreciable difference in liver glycogen concentration of the treated and control groups. However, in our acute experiments it was found that glycogenic activity is more likely to be discerned if tested on a liver almost devoid of glycogen; it is therefore quite possible that some degree of glycogen deposition may have occurred as a result of cystine feeding, but the experimental conditions were not such as to permit its observation. On the other hand, in the experiments of Foldes glycogen accumulation was of such magnitude as to be comparable with von Gierke's disease; certainly no such histological changes are possible with a liver glycogen concentration of only 1.3%. It may be concluded, therefore, that cystine, cysteine or glutathione sulfur under these experimental conditions exert no glycogenic effect in addition to that of the amino acid itself.

It is of particular interest that glutathione is apparently not converted

to glycogen on injection into the adrenalectomized animal; in the intact, partially glycogen-depleted animal an actual drop of liver glycogen was observed. Other investigations (Goldzieher, Rawls and Goldzieher, 1953) have shown that the sulfhydryl of the injected glutathione is not detectable as free SH in the liver, where much of it is supposed to accumulate. Yet tracer studies with C^{14}, N^{15} or S^{35}-labelled material (Bloch, 1949; Waelsch and Rittenberg, 1941; Anderson and Mosher, 1951) indicate that this compound has intense metabolic activity. For example, half the glycine in glutathione is turned over in less than 8 hours (Waelsch and Rittenberg, 1941). This would suggest the presence of mechanisms designed to protect this particular compound in contradistinction to other tripeptides which may be liberated in the process of degrading ingested or endogenous proteins.

SUMMARY

Rats were partially depleted of liver glycogen by fasting for 48 hours then serine, cysteine or glutathione was given orally or intraperitoneally. Neither serine nor cysteine altered the liver glycogen level; glutathione caused a significant fall. With maximum depletion of liver glycogen (maximum a 24 hour fast) cysteine caused an increase of liver glycogen.

Oral administration of *l*-cystine for 25 days did not cause significant glycogen storage.

In fasted-adrenalectomized animals, serine and cysteine caused glycogen storage of comparable magnitude, while glutathione caused none.

It is concluded that amino acid sulfur (as sulfhydryl or disulfide) under these conditions has no specific effect on liver glycogen deposition, either in normal or adrenalectomized animals. The response to glutathione differs from that of the amino acids tested. The degree of glycogen depletion affects the response to administered amino acids.

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THE ADRENAL CORTEX AND SULFUR METABOLISM: THE EFFECT OF ACTH ON THE SULFHYDRYL CONCENTRATION OF RAT ADRENAL, LIVER AND MUSCLE[1]

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AS EARLY as 1909 it was noted that the adrenal cortex was unexpectedly active in sulfur metabolism: both its sulfur content and its ability to store such compounds were greater than those of other tissues studied (Aufrecht and Diesing, 1909; Loeper *et al.*, 1926). In 1928 it was shown that treatment of rats and rabbits with colloidal sulfur resulted in adrenal cortical hypertrophy and sulfur storage, together with massive accumulation of liver glycogen (Lurie, 1928; Foldes, 1928). At that time, however, the changes in these two organs could not be interpreted, for the relation between liver glycogen deposition and 11-oxycorticosteroids was as yet unsuspected.

In recent years, further evidence has accumulated to show a relation between sulfur metabolism and adrenal cortical function. Studies with S^{35} for example, have confirmed the great avidity of the cortex for cystine sulfur (Lee *et al.*, 1951). Recent studies in our laboratory (Goldzieher *et al.*, in press) have demonstrated a rapid accumulation of SH in the adrenal cortex following the administration of cysteine. Conversely, ACTH and cortisone have been shown to influence blood levels of sulfhydryl (Hess *et al.*, 1951; Goldzieher *et al.*, in press) in animals and man. The incorporation of S^{35} into regenerating connective tissue is inhibited by cortisone in direct proportion to its concentration in the tissue (Layton, 1951). The urinary excretion of SH and SS sulfur is increased by the administration of ACTH (Kinsell *et al.*, 1950).

The relation of the adrenal cortex to sulfur metabolism has been under investigation by us for some time; the present report deals only with the effect of ACTH on the sulfhydryl content of the adrenals, and in comparison, with that of other tissues such as liver and muscle.

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MATERIALS AND METHODS

Adult male CFW rats, weighing 200 to 250 Gm. were used throughout. They were maintained on a diet of Purina Fox Checkers and water *ad lib.* Food was withheld during the night prior to treatment.

ACTH[3] was prepared just before use in the single-dose experiments and daily for chronic studies. The quantities designated are in terms of International Standard. Injections of ACTH were given intramuscularly, and the animals were killed by a blow on the head three hours after the last injection. Both adrenals were dissected rapidly, freed of adherent tissue, enclosed in parafilm, and weighed to the nearest 0.1 mg. Approximately 100 mg. portions of liver and diaphragm were prepared in the same way. The tissues were ground to an emulsion in an all-glass Ten Broeck tissue grinder in 2.5 or 5.0 ml. 2.5% sulfosalicylic acid containing 0.5% disodium ethylene diamine tetraacetate. The emulsions were centrifuged for 20 minutes and the supernatant used for titration. The sulhydryl determinations were carried out by the amperometric method described by Benesch and Benesch (1950) and by Weissman, Schoenbach and Armistead (1950), as modified by us (Goldzieher and Epstein, in press) to increase the specificity and reproducibility of the method.

The dosage and duration of ACTH treatment is given in the following tables. The standard deviation and statistical significance of the data were evaluated by means of the t-test, using the formulas corrected for small groups.

RESULTS

Adrenal nonprotein sulfhydryl. The changes in adrenal sulfhydryl with varying dosage schedules of ACTH are shown in Table 1. The control value of 14.9 ± 1.6 mg.% is the result of determinations on 42 animals taken in small groups over a considerable period of time. The variation in normal adrenal sulfhydryl concentration, 10.8%, is relatively small.

The smallest dose of ACTH used, 0.1 mg., produced an increase in adrenal sulfhydryl concentration of 45%; this was of a high order of statistical significance ($p. = \ll 0.01$) and differed considerably from the response of the liver (Table 2) to the same dose. A similar reaction could not be obtained on repeating an 0.1 or 0.3 mg. dose at 8-hour intervals over 1 day,

TABLE 1. CHANGES IN RAT ADRENAL SULFHYDRYL

Treatment	Number of animals	SH concentration in mg./100 Gm. tissue (average ±st. dev.)	Significance	
			(t-value)	P
Control	42	14.9±1.6		
ACTH, 0.1 mg.; killed after 3 hrs.	6	21.6±4.1	4.8	<0.01
ACTH, 0.1 mg. q. 8 hrs. ×3	6	15.5±2.9	0.7	
ACTH, 0.3 mg. q. 8 hrs. ×3	5	15.2±2.9	1.0	
ACTH, 4.0 mg.	4	12.4±3.4	3.4	<0.01
ACTH, 4.0 mg. q. 12 hrs. ×7	4	13.1±2.1	2.9	<0.01
ACTH, 0.3 mg. q. 12 hrs. ×25	3	11.7±0.1	4.1	<0.01
ACTH as above, then no Rx for 5 days	3	11.3±2.3	4.2	<0.01

[3] Lot 212-103, supplied through the courtesy of Armour & Co.

TABLE 2. CHANGES IN RAT LIVER SULFHYDRYL

Treatment	Number of animals	SH concentration in mg./100 Gm. tissue (average ±st. dev.)	Significance	
			(t-value)	P
Control	4	31.3±3.5		
ACTH, 0.1 mg., killed after 3 hrs.	3	29.4±2.5	0.7	
ACTH, 0.3 mg. q. 8 hrs. ×3	2	24.1±0.7	2.4	0.05
ACTH, 0.3 mg. q. 12 hrs. ×25	3	23.3±2.6	3.0	0.03
ACTH as above, then no Rx for 5 days	3	24.0±2.9	2.6	0.05

for such treatment did not produce changes in adrenal sulfhydryl concentration. Large and relatively unphysiologic doses (4 mg.) or multiple doses produced a consistent and statistically significant depression of adrenal sulfhydryl concentration of the order of 12 to 24%. It is thus possible that the lack of change in adrenal sulfhydryl concentration during treatment with 0.3 to 0.9 mg. ACTH per day represents a balance point or cancellation between the SH-augmenting effect of a small dose and the SH-depressant effect of larger or multiple doses. The adrenal sulfhydryl depletion caused by 12 days of ACTH therapy was not restored after a 5-day recovery period, although the adrenal weights were once more within normal limits.

Liver nonprotein sulfhydryl. With liver tissue, the response to ACTH was not biphasic (Table 2). The small doses which produced a rise or no change in adrenal sulfhydryl concentration, caused no change and a fall, respectively, in the sulfhydryl concentration of liver tissue. Prolonged ACTH therapy caused a significant fall in the liver nonprotein sulfhydryl, just as in the adrenal, and again there was no significant recovery by the fifth day after the end of a 12-day course of ACTH treatment. These observations do not coincide with the findings of Ingbar, Otto and Kass (1951), who administered 9 mg. ACTH per day to rats for 3 and 10-day intervals, and found no change in liver nonprotein sulfhydryl after 3 days, and an actual increase after 10 days.

Muscle (diaphragm) nonprotein sulfhydryl. A few experiments were performed on rat muscle tissue (Table 3). Three or more injections of ACTH produced a significant depletion of nonprotein sulfhydryl; as with adrenal and liver tissue, there was no complete return to pretreatment levels within 5 days after the end of chronic ACTH treatment. The muscle, however, showed more of a tendency to return to normal levels than did the other tissues.

DISCUSSION

Several points are brought out by these observations. The effect on the adrenal of small doses of ACTH is entirely different from that of large doses or chronic treatment; it also differs from the response of the liver. It

TABLE 3. CHANGES IN RAT MUSCLE (DIAPHRAGM) SULFHYDRYL

Treatment	Number of animals	SH concentration in mg./100 Gm. tissue (average ±st. dev.)	Significance	
			(t-value)	P
Control	8	9.4±2.1		
ACTH, 0.3 mg. q. 8 hrs. ×3	3	6.6±0.9	2.2	0.05
ACTH, 0.3 mg. q. 12 hrs. ×25	3	4.0±1.1	4.1	<0.01
ACTH as above, then no Rx for 5 days	3	5.9±0.6	2.8	0.02

is interesting to note that this rise in adrenal nonprotein sulfhydryl coincides closely with the time at which a *drop* in blood sulfhydryl is observed in patients receiving continuous infusions of ACTH (Goldzieher *et al.*, in press).

With larger doses, the adrenal seems to behave in a nonspecific manner, for all the tissues studied undergo a depletion of nonprotein sulfhydryl. The fact that this depletion persists for some time after the end of ACTH therapy, and is found in tissues of highly diverse structure and function, indicates that it cannot be considered as a specific effect of ACTH in the same sense as the changes in adrenal ascorbic acid or adrenal cholesterol. Studies are now in progress to determine more precisely the nature of the sulfhydryl-bearing compound which is readily depleted but only slowly restored.

Anderson *et al.* (1951) have commented on the possibility that ACTH and cortisone exert their clinical effects by interfering with the sulfhydryl groups of enzyme systems. It must be noted that the sulfhydryl-bearing substances which we have studied are non-protein in nature, and are therefore not enzymes; however, they may represent the reservoir of freely-available sulfhydryl from which the SH-groups of the enzymes are replenished after engaging in the processes of oxidation-reduction associated with metabolic phenomena.

SUMMARY

Single small doses of ACTH produce what appears to be a specific reaction: a rise in the adrenal nonprotein sulfhydryl in 3 hours. Larger doses or prolonged treatment with ACTH produce a drop in nonprotein sulfhydryl which is not specific, for comparable changes also occur in liver and muscle tissue. The depletion is not restored in any of these tissues within 5 days following the end of prolonged ACTH administration.

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COMPARISON OF OVARIAN AND PITUITARY HORMONES FOR MAINTENANCE OF PREGNANCY IN PYRIDOXINE-DEFICIENT RATS[1]

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PREVIOUS studies have shown that the addition of a potent B6-antagonist, desoxypyridoxine, to a purified B6-deficient diet will result in the rapid production of a B6-deficiency in adult rats (Nelson and Evans, 1948, 1951). Acrodynia of the paws, a characteristic sign of the deficiency, and marked reproductive disturbances, namely a high incidence of resorptions, occurred after 2–3 weeks of the deficient diet. Pregnancy was maintained in such B6-deficient rats by administration of both estrone and progesterone but not by either hormone separately (Nelson, Lyons, and Evans, 1951), indicating some similarity between these B6-deficient rats and hypophysectomized-oophorectomized rats in which pregnancy can be maintained by the combination of the same hormones at the same dosage levels (Lyons, 1943). To determine whether pituitary or ovarian dysfunction is involved in the hormonal inadequacies of the B6-deficient rat, the three pituitary gonadotrophins which maintain pregnancy in the hypophysectomized rat have been tested in the vitamin-deficient rats. These three pituitary gonadotrophins are FSH, ICSH or LH, and MH (mammotrophin or lactogenic hormone, Lyons, Simpson and Evans, 1943).

EXPERIMENTAL METHODS

Stock rats of the Long-Evans strain were placed on the B6-deficient diet containing the antagonist at 80–85 days of age. This diet was the same as that used in all previous

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studies. It consisted of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated cottonseed oil (Crisco or Primex), 4% salts,[2] and 0.5 mg.% desoxypyridoxine. Crystalline vitamins per kilogram of diet were: 300 μg. d-biotin, 5 mg. 2-methyl-1, 4-naphthoquinone, 5 mg. thiamine HCl, 5.5 mg. pteroylglutamic acid, 10 mg. riboflavin, 10 mg. p-aminobenzoic acid, 20 mg. nicotinic acid, 50 mg. d-calcium pantothenate, 400 mg. inositol, and 1.0 gm. choline chloride. Each rat received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vitamin A, 115 chick units vitamin D, 6 mg. synthetic alpha-tocopherol, and 650 mg. corn oil (Mazola).

After 17 days of deficiency, the rats were bred with normal males and divided into equivalent groups as judged by body weight on the day of breeding. This experimental procedure has been shown to result consistently in 90–100% resorptions[3] in the deficient rats which usually average 21 days of deficiency prior to breeding (Nelson and Evans, 1951). One experiment was carried out in which animals with a longer period of deficiency, 28 days, was used; these rats were brought into estrus by means of one injection of 10 units pregnant mare serum (PMS). Vaginal smears were examined daily during gestation for the presence of erythrocytes, the sign that implantation had occurred. All rats were weighed at regular intervals. To minimize the question of placental hormone production, known to be so important in the latter part of pregnancy, all animals were autopsied 13 days after breeding; implantation sites, fetuses, and ovaries were carefully examined.

Injections were started 3 days after finding sperm in the vaginal smear. The steroid hormones used, estrone and progesterone, were injected subcutaneously in 0.2–0.3 cc. sesame oil. The anterior pituitary hormones used were dissolved in distilled water or physiological saline and neutralized. An FSH-ICSH combination capable of causing estrin production in the hypophysectomized rat and previously used successfully for maintenance of pregnancy in such animals (Lyons *et al.*, 1943; Lyons, 1943) was used together with both impure and purified MH preparations, which varied in activity from 10–30 I.U./mg.

Since it was possible to breed only 50% or less of the deficient animals in any large group of 30–60 rats, 5 separate experiments were carried out, 4 experiments with the standard period of 21 days of deficiency prior to breeding and one experiment with the longer period of deficiency, 28 days.

RESULTS AND DISCUSSION

Table 1 summarizes the results of testing the pituitary gonadotrophins in the B6-deficient rat. With 21 days of deficiency prior to breeding, the uninjected group showed 94% resorptions with early appearance of erythrocytes in the vaginal smear (day 10) due to early resorbing sites. The group receiving estrone and progesterone showed approximately 80% maintenance of pregnancy with normal time of appearance of vaginal erythrocytes on day 12. In contrast, the group injected with the pituitary gonadotrophins, FSH-ICSH and MH, exhibited partial maintenance of pregnancy,

[2] Salts No. 4 of Hegsted *et al.* (1941).

[3] The normality of reproductive performance in pair-fed controls supplemented with high levels of pyridoxine has previously demonstrated that food restriction is not a factor in the reproductive upsets occurring in this vitamin deficiency (Nelson and Evans, 1951).

only ¼ of the group having living young, with early appearance of vaginal erythrocytes on day 10.

To determine whether one particular hormone or all three gonadotrophins were involved, the combination of a single ovarian hormone together with the appropriate gonadotrophin(s) was next tried. Lyons (1951) has shown that when the FSH-ICSH preparation to stimulate estrogen production was injected together with progesterone, pregnancy was maintained in rats hypophysectomized after breeding; the combination of estrone with MH also maintained pregnancy in such animals (Lyons *et al.*, 1943).

In the B6-deficient rat the use of estrone together with MH gave es-

TABLE 1. EFFECT OF PITUITARY GONADOTROPHINS ON REPRODUCTION OF PYRIDOXINE-DEFICIENT RATS

Exptl. group[1]	Rats bred	Wt. change during gestation	Vaginal RBC[2]	Implantations	Implants with resorptions	Implants with living embryos	Implantation sites	
							Av. No.	Normal No.[3]
	No.	gms.	day	%	%	%		
21 Days of Deficiency Prior to Breeding								
Uninjected	17	−9	10.1	94	94	6	9.1	
120–160 μg. FSH-ICSH +70–125 I.U. MH	15	+8	10.2	100	73	27	10.0	5.2
0.5 μg. Estrone +96–125 I.U. MH	9	+5	10.2	100	78	22	10.1	3.5
120 μg. FSH-ICSH +4 mg. Progesterone	10	−4	11.0	100	90	10	10.7	
0.5 μg. Estrone +4 mg. Progesterone	29	−2	12.1	97	21	79	9.0	5.2
28 Days of Deficiency Prior to Breeding[4]								
Uninjected	9	−3	9.0	22	100	0	8.0	
200 μg. FSH-ICSH +75 I.U. MH	9	−6	11.4	78	71	29	7.1	4.0
0.5 μg. Estrone +4 mg. Progesterone	9	+1	13.0	89	0	100	5.8	3.5

[1] Injections were started 3 days after breeding. All animals were autopsied 13 days after breeding.
[2] This column shows the average time in days for the first appearance of erythrocytes (RBC) in the vaginal smear.
[3] This column gives the number of living embryos per litter, i.e. the number of normal implantation sites in rats with maintenance of pregnancy. Values are given only for groups in which at least 2 rats maintained pregnancy.
[4] These rats were brought into estrus by means of one injection of pregnant mare serum (10 units).

sentially the results obtained by the use of the three pituitary gonadotrophins, namely partial maintenance of pregnancy, showing that MH stimulated ovarian production of progestin in only a few animals. The use of FSH-ICSH together with progesterone was not as beneficial, only 10% of the animals showing maintenance of pregnancy, an incidence not significantly different from that shown by animals uninjected or injected with progesterone alone (Nelson, Lyons and Evans, 1951). This may be interpreted as a failure of combined FSH-ICSH to stimulate the ovarian production of estrin in these B6-deficient rats and suggests ovarian dysfunction since the same dosage level of this hormone preparation will cause estrin production in normal immature rats and even lower doses will do so in hypophysectomized rats.

An additional experiment was carried out to check on the beneficial effects of the pituitary gonadotrophins. A longer period of deficiency was

used so that the deficient animals were brought into estrus by a single injection of 10 units of PMS. All animals responded with an estrous vaginal smear and 55% of the group were successfully bred. Rats receiving no hormone injections during gestation showed a marked failure of implantation due to the longer period of deficiency prior to breeding; only 22% of the group implanted, in contrast to the usual 90–100% implantations, and all implantations resorbed. Rats injected with estrone and progesterone exhibited the usual favorable response, i.e., 100% implantations with 100% maintenance of pregnancy. Rats injected with the three pituitary gonadotrophins showed 78% implantations and partial maintenance of pregnancy; 29% of the group had living young. The marked improvement in implantation in contrast to the findings in the uninjected group shows the beneficial effects of injected gonadotrophins and indicates some pituitary dysfunction. Nevertheless, the failure to maintain pregnancy in a larger proportion of the animals would seem to clearly show ovarian dysfunction.

Whether the lack of ovarian response to injected gonadotrophins may be corrected by higher dosage levels, by a different hormone balance, or by supplementation with other pituitary hormones is not known. In adult rats hypophysectomized after breeding, daily injections of 60 I.U. MH and 60–200 μg. FSH-ICSH were successful in maintaining pregnancy (Lyons *et al.*, 1943; Lyons, 1951). In this study daily dosage levels of 120–200 μg. FSH-ICSH and 70–125 I.U. MH were used without the same degree of success. Although the hypophysectomized rat is considered to be more sensitive than an intact animal to pituitary hormones, it would usually be assumed that a vitamin-deficient animal with an intact pituitary would have some circulating gonadotrophin present. Such an animal would presumably require a decreased amount rather than an increased amount of gonadotrophin. Further studies on dosage levels are definitely indicated and are in progress.

The inadequate ovarian response to pituitary gonadotrophins brings up the question of possible placental dysfunction in these deficient animals. It was previously pointed out that although pregnancy could be maintained by estrone and progesterone in these deficient animals the number of living young per litter was less than half the normal value (Nelson, Lyons and Evans, 1951). The same results were obtained in this study, regardless of whether the injected hormones were ovarian or hypophysial in origin.

SUMMARY

Under the experimental conditions anterior pituitary gonadotrophins maintained pregnancy in approximately 25% of pyridoxine-deficient animals whereas ovarian hormones maintained pregnancy in 80–100% of such animals. This indicates that dysfunction of both the pituitary and the

ovary is involved in the hormonal inadequacies of these vitamin-deficient animals.

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THE PROGESTERONE CONTENT OF HUMAN PLACENTAL BLOOD[1]

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AN INVESTIGATION of the ketonic lipides of placental blood was undertaken in conjunction with our recent work (Pearlman and Cerceo, 1952a, 1952b) on the isolation of progesterone[2] and related C21 steroids from human placental tissue. In this study, however, progesterone could not be isolated because it was present in minute amount only. An estimate was made of the progesterone level of pooled placental blood by ultraviolet spectroscopy of the ketonic fraction following chromatography and countercurrent distribution.

EXPERIMENTAL

Collection, extraction, and preliminary fractionation of blood.—Full term placentas were obtained as soon as possible after parturition. Each specimen was perfused with 200 cc. of 0.9% NaCl through the umbilical vein following removal of clots of adhering maternal blood. The blood perfusate was immediately extracted and then partitioned in the manner outlined in Diagram 1 to yield the ketonic fraction.

Estimation of the progesterone content of the ketonic fraction.—The method used was that described by Pearlman and Cerceo (1952b) for estimation of the progesterone content of placental tissue[3] and requires measurement of the optical density at 240 mμ (maximal absorption due to α, β unsaturated ketones) following an 8 transfer distribution of the ketonic material between petroleum ether and 70% methanol; in the present study however, the volumes of the immiscible solvents were reduced to one-fifth.

The progesterone level of the blood perfusates of four placentas were individually estimated and found to fall within the range of 0.75 to 0.86 μg. of hormone per cc.; the pooled perfusates obtained from six other placentas were estimated to contain 0.69 μg. of progesterone per cc. These values are

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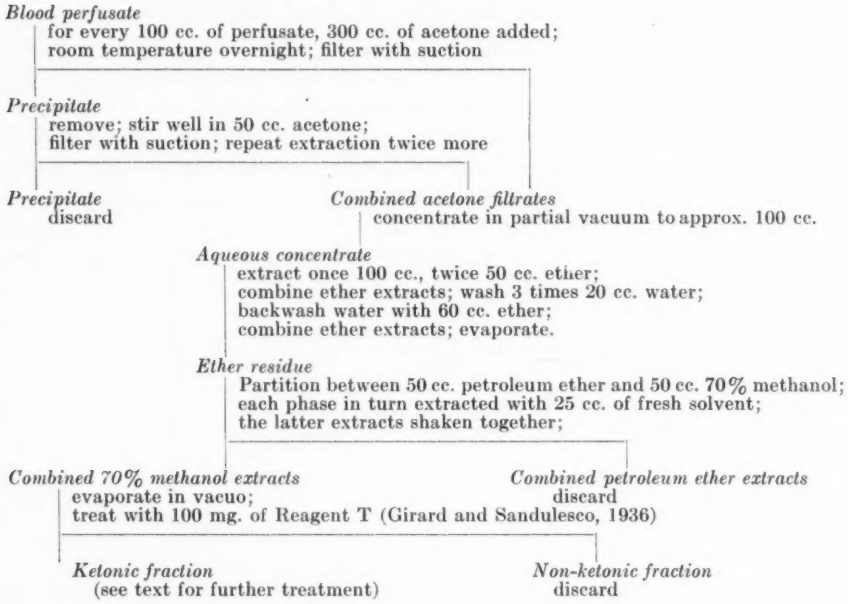
[1] This investigation was supported in part by a research grant (RG-2567 C) from the National Institutes of Health, Public Health Service.

[2] Salhanick *et al.* (1952) have also announced the isolation of progesterone from human placenta; Diczfalusy (1952) simultaneously reported indirect evidence for the presence of this hormone.

[3] The procedure recently described by Diczfalusy (1952) is very similar but more refined, requiring a 24 transfer distribution between petroleum ether and 34.5% ethanol.

DIAGRAM 1. Extraction and Fractionation of Placental Blood


Blood perfusate
for every 100 cc. of perfusate, 300 cc. of acetone added;
room temperature overnight; filter with suction
Precipitate
remove; stir well in 50 cc. acetone;
filter with suction; repeat extraction twice more
Precipitate
discard
Combined acetone filtrates
concentrate in partial vacuum to approx. 100 cc.
Aqueous concentrate
extract once 100 cc., twice 50 cc. ether;
combine ether extracts; wash 3 times 20 cc. water;
backwash water with 60 cc. ether;
combine ether extracts; evaporate.
Ether residue
Partition between 50 cc. petroleum ether and 50 cc. 70% methanol;
each phase in turn extracted with 25 cc. of fresh solvent;
the latter extracts shaken together;
Combined 70% methanol extracts
evaporate in vacuo;
treat with 100 mg. of Reagent T (Girard and Sandulesco, 1936)
Combined petroleum ether extracts
discard
Ketonic fraction
(see text for further treatment)
Non-ketonic fraction
discard

undoubtedly overestimated because the ketonic material in the peak tube (separatory funnel no. 4) in the countercurrent distribution did not give a well defined absorption curve characteristic of α, β unsaturated ketones; further purification of this fraction was needed.[4] Accordingly, the ketonic residues from separatory funnel no. 4, obtained in the countercurrent distribution of the above perfusates (total 1715 cc., ten placentas), were combined to yield 4.4 mg. of solids containing at the very most 384 μg. of progesterone. This material was dissolved in 0.2 cc. of dry ether, adsorbed on a narrow column containing 200 mg. of alumina[5] (Harshaw) previously wetted with the same solvent, and eluted with 20 cc. of dry ether. The eluate gave an ultraviolet absorption curve typical of an α, β unsaturated ketone, and was estimated to contain 186 μg. of progesterone;[6] counter-

[4] Reynolds and Ginsburg (1942) have similarly encountered pigments with nonspecific ultraviolet absorption which interfered with their attempts to estimate normal serum progesterone levels in another connection. In our experiments, such pigments were found to accumulate chiefly in the end separatory funnels in the countercurrent distribution procedure.

[5] The preparation is described in footnote 4 (Pearlman and Cerceo, 1952a).

[6] Chromatography of the same quantity of crystalline progesterone under identical conditions resulted in the recovery of 75% of the hormone according to measurements of the optical density at 240 mμ.

current distribution (Fig. 1) indicated a content of 182 μg. of progesterone (or of an α, β unsaturated ketone with a partition coefficient identical with that of progesterone). Recalculation, on the basis of the latter determination, of the progesterone level of the pooled blood perfusates (total 1715 cc.) gave a value of 0.39 μg. of the hormone per cc.

The ketonic residues from separatory funnel no. 8 in the original countercurrent distribution were similarly combined, representing the pooled perfusates from ten placentas. This material, from its position in the counter-

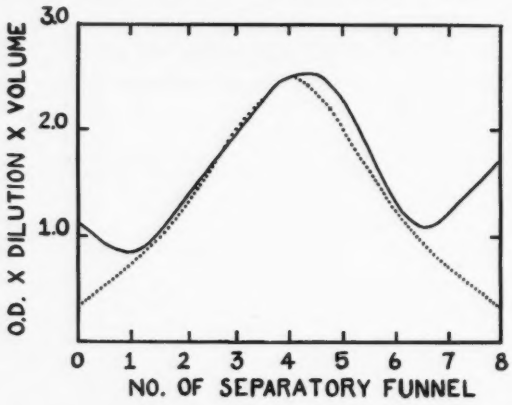


FIG. 1. Countercurrent distribution of chromatographed residue obtained from separatory funnel no. 4 (see text for further details). Solvent system, petroleum ether-70% methanol (volume ratio 3:1). Optical density (O.D.) measured at 240 mμ; solvent, absolute ethanol. Experimentally found curve, solid line; calculated curve for progesterone, dotted line.

current distribution scheme, might be expected to contain α, β unsaturated ketones more polar than progesterone. However, the presence of interfering pigments could not be entirely eliminated by chromatography and further work on this fraction was abandoned.

DISCUSSION

The analytical procedures described herein were not designed for the routine examination of small specimens of blood, although these methods might be adapted for this purpose. Our aim, rather, was to obtain a sufficiently large quantity of placental blood (and hence resort to perfusion of the placenta) in order to carry out an exploratory examination of the ketonic material by physico-chemical means.

The data indicate that the progesterone concentration of pooled placental blood is of the order of 0.4 μg. of hormone per cc., a conservative estimate inasmuch as the blood obtained by perfusion underwent some dilu-

tion and also, part of the progesterone (roughly 25%) was lost by chromatography. It is clear that the placental blood could have contributed in small part only to the total progesterone content of our placental extracts (Pearlman and Cerceo, 1952b), a matter which was of some concern since, in that study, the blood had not been removed by perfusion of this highly vascular organ.

Although the absolute concentration of progesterone in placental blood is quite low, it is high by comparison with that in maternal blood, which is less than 0.1 μg. of progesterone per cc. (Butt *et al.*, 1951). The latter value, obtained by polarographic methods, is at variance with the higher values reported by Hooker and Forbes (1949) who employed bio-assay techniques.

A comparison of our data on placental blood with those of Butt *et al.* (1951) are of interest. The latter investigators found 1.3 and 0.3 μg. of progesterone per cc. in two specimens, respectively, of human placental blood obtained from the umbilical cord but in three other specimens, no progesterone (i.e., less than 0.1 μg.) could be detected; however, there was polarographic evidence for the presence of a Δ^4-3 ketosteroid but not of a 20-ketosteroid (the progesterone molecule contains both groups). The latter observation stimulated our interest but our study provides no information on this point.

SUMMARY

The progesterone level of pooled placental blood is conservatively estimated to be 0.4 μg. of hormone per cc. This calculation is based on measurements of the optical density at 240 mμ. of the ketonic fraction following chromatography and countercurrent distribution.

Acknowledgment

The authors are indebted to the Jefferson Medical College Hospital for permission to collect placental blood.

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DETECTABLE AMOUNTS OF ACTH IN BLOOD FOLLOWING SALICYLATE ADMINISTRATION

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ALTHOUGH a tabulation (Hailman, 1952) of various physiological responses induced by cortisone, ACTH, and salicylate (SAL) suggests an ACTH mechanism of SAL effect, an increase in circulating ACTH following SAL has not been demonstrated. Because of our general interest in the effect of salicylate upon adrenal function and the probability that some, at least, of these effects may be mediated through the pituitary gland we have undertaken to determine, by the transfusion technique of Farrell and McCann (1952), whether SAL administration stimulates release of ACTH into the circulation.

Female Sprague-Dawley rats (180–210 gm.) were injected intraperitoneally with 0.6 ml. of sodium salicylate solution containing the equivalent of 30 mg. of salicylic acid/100 gm. body weight. After varying intervals the rats were etherized and the external jugular vein quickly exposed. Three ml. of blood were withdrawn into a heparinized syringe and immediately transfused into the corresponding vein of an etherized, hypophysectomized Sprague-Dawley rat of approximately the same weight. This transfusion required about 1 or 2 minutes. The hypophysis had been removed from the recipient rat usually 2 days but not more than 3 days earlier. The left adrenal of the recipient rat was removed just before, and the right adrenal one hour after, the transfusion. Each adrenal was assayed for ascorbic acid without delay, using the method of Roe and Kuether (1942). As in the method of Sayers, *et al.* (1948), the difference in ascorbic acid concentration between the left and right adrenals of the recipient was taken as an index of ACTH level in the blood of the donor rat. The lowering of adrenal ascorbic acid is an effect thought to be specifically produced by ACTH under such conditions. To check both the effect of SAL itself and the operative procedures on the hypophysectomized rats, 0.2 ml. of 5.8% Na SAL was added to 2.8 ml. of blood obtained from donor rats injected 8–10 minutes earlier with isotonic saline solution instead of Na SAL; and this blood mixture was assayed similarly for ACTH activity by injection into control hypophysectomized rats.

SAL levels and adrenal ascorbic acid depletion in the donor rats were

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TABLE 1. ADRENAL ASCORBIC ACID (AAA) DEPLETION IN HYPOPHYSECTOMIZED RATS ONE HOUR AFTER TRANSFUSION OF 3 ML. OF BLOOD FROM SAL-INJECTED RATS

Time after SAL injection of donor rats	No. of recipient rats	Decrease in AAA in hypophysectomized recipient rats	
minutes		mg./100 gm. of adrenal tissue	%
control	7	7 ± 20*	2
5	2	−3	0
15	8	50 ± 10*	12
30	3	38	10
60	4	31 ± 17*	8
85	2	12	3

* ± values are standard errors, calculated where sufficient numbers of animals were used for significant statistical treatment.

determined in order to correlate them with the adrenal ascorbic acid reduction in the recipient rats. These were compared also with control donors receiving isotonic saline instead of SAL injections. The method of Smith and Talbot (1950), involving the use of the Folin-Ciocalteu reagent for phenols, was used in the estimation of the plasma SAL levels.

RESULTS AND DISCUSSION

The dosage of SAL used was about ½ of the lethal dose (Waddell, 1911). After injection the rats showed no obvious discomfort and exhibited no unusual behavior. The rats were gentle and exhibited very little excitement when injected, thus minimizing any ACTH rise resulting from the mere handling of the animals.

The results presented in Tables 1 and 2 indicate that very soon after the SAL is injected, there is an increase in circulating ACTH as reflected by a decrease in both donor and recipient adrenal ascorbic acid. Since injected ACTH is rapidly destroyed, the results in terms of percentage decrease in adrenal ascorbic acid are less striking in the recipients than in the donors. Despite the continued high blood level of SAL in the donor the

TABLE 2. ADRENAL ASCORBIC ACID (AAA) AND PLASMA SAL LEVELS IN DONOR RATS INJECTED WITH 30 MG. SAL/100 GM. BODY WEIGHT

Time after injection of donor rats	Type of injection	No. of donor rats	AAA	AAA decrease	Donor plasma SAL levels minus blank
minutes			mg./100 gm. of adrenal tissue	%	mg./100 ml.
15	SAL	10	375	19	57.6
15	Saline	5	463		0
60	SAL	5	232	50	55.6
60	Saline	5	463		0
120	SAL	5	158	54	40.6
120	Saline	5	347		0

ACTH activity appears to decline from an initial maximum, presumably as the preformed donor ACTH stores are consumed.

SUMMARY

The ACTH titer in blood of SAL-injected rats has been shown to rise very soon after the injection, as assayed by transfusion into hypophysectomized rats of blood from SAL-injected rats.

Acknowledgment

The advice of Dr. J. S. Davis on surgical procedure is gratefully acknowledged. The authors also wish to thank the S. E. Massengill Co., of Bristol, Tennessee, for providing some of the animals and chemicals used here. The hypophysectomized rats were obtained from Hormone Assay Laboratories, Chicago, Illinois.

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GLUCURONIDASE HYDROLYSIS FOR PREGNANEDIOL ASSAYS[1,2]

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THE great interest shown in pregnanediol assays is based upon a proof of relationship between pregnanediol and the corpus luteum hormone —progesterone (Marrian 1949). The methods for assaying pregnanediol in urine are: Firstly the method of Venning (1937, 1938) in which the sodium pregnanediol glucuronidate (NaPG) of the urine is assayed gravimetrically; and secondly the hydrolysis of the pregnanediol conjugate and the assay of the pregnanediol (Astwood and Jones, 1941; Talbot *et al.*, 1941). In 1948 Sommerville, Gough, and Marrian modified the test in order to obtain a sensitivity of 2.5 mg./day. This paper introduces the hydrolysis of urines by calf spleen glucuronidase. This method has been found to give excellent results for assaying less than 0.50 mg. pregnanediol /24 hrs.

MATERIALS

All chemical reagents were chemically pure. The toluol was redistilled. The sodium pregnanediol glucuronidate was freed of sodium pregnaneolone glucuronidate by the procedure of Sutherland and Marrian (1947). An all glass filter funnel similar to that described by Engley (1952) and containing a sintered glass filter of medium porosity was used to filter off the pregnanediol before assay. The glucuronidase was prepared and assayed according to the procedure of Cohen (1951). The normal male urine used in these experiments was pooled urine. The pregnancy urines were collected from individual girls from 32 to 38 weeks of pregnancy. The pregnanediol assays with sulphuric acid were carried out essentially by the procedure of Talbot *et al.* (1941).

EXPERIMENTAL

The Hydrolysis of NaPG and the Purification of Pregnanediol

The technique described by Cohen (1951) for the hydrolysis of pregnanediol glucuronide was employed. A modified technique of Sommerville

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[1] This work was aided by grants from the National Institutes of Health, United States Public Health Service and from the Graduate School of the University of Minnesota.

[2] This paper was read before the Endocrine Society on June 5, 1952.

[3] The work reported herein is taken largely from a thesis being presented by M. M. Goldfine in partial fulfillment for the degree of Master of Arts by the University of Minnesota Graduate School.

et al. (1948) was employed for the purification of pregnanediol. This scheme is outlined in Chart I.

RATE OF HYDROLYSIS

The hydrolysis of late pregnancy urine gives the same type of curve as did that employing pure NaPG (Cohen 1951). Hydrolysis is complete within 5 to 6 hours, indicating that urine NaPG is about twenty times more labile to glucuronidase hydrolysis than are most steroids. These results are illustrated in Figure 1.

CHART I. Procedure for the hydrolysis of NaPG and purification of urinary pregnanediol.

URINE
100–500 cc.
Sterilize by boiling 10 minutes
Cool, adjust to pH 4.5 (0.1 N) with acetate buffer +1000 units glucuronidase, incubate 6 hours to overnight

Extract with 2×1/2 vols. toluene (emulsions broken by configuration)

——————————URINE RESIDUE

TOLUENE
Wash with 2×1/10 vol. N. NaOH. 1×1/10 vol. H_2O

To dryness *in vacuo*
Dissolve residue in 15–20 cc. hot ETOH, +1–2 mg. charcoal; filter (Whatman 12.5 cm. No. 12 Filter Paper)

——————————CHARCOAL

FILTRATE
Concentrate to 5 cc. volume; +20 cc. hot 0.1 N NaOH. and incubate at 37° for 2–4 hrs. Filter (Engley funnel)

——————————FILTRATE

PRECIPITATE
Dissolve in 15–25 cc. ETOH

ASSAY ALIQUOTS

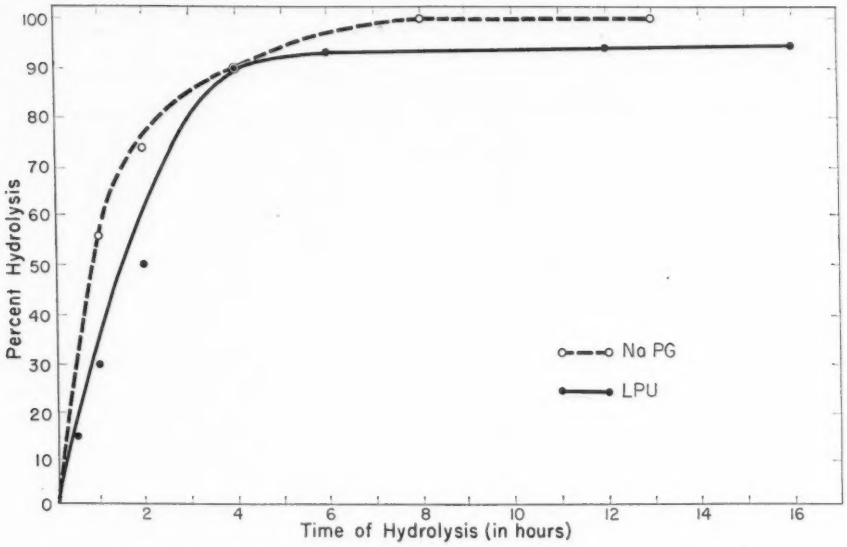

FIG. 1. The hydrolysis of pure NaPG and of late pregnancy urine (L.P.U.) with 1000 units glucuronidase.

RECOVERIES OF PURE NaPG ADDED TO MALE URINE

The data outlined in Table 1 illustrates that good recoveries can be obtained down to 0.04 mg. of pregnanediol per 100 cc. urine.

RECOVERIES OF PREGNANEDIOL FROM HUMAN PREGNANCY URINE

In Table 2 the accuracy of the pregnanediol assay is further shown. It can be seen from this table that glucuronidase liberates as much preg-

TABLE 1. AMOUNT OF PREGNANEDIOL RECOVERED FROM MALE URINE TO WHICH NaPG HAD BEEN ADDED BY GLUCURONIDASE (Gl) HYDROLYSIS

Pregnanediol added as NaPG	Solvent	Gl hydrolysis (corrected for control)
3.40 mg.	100 cc. NMU P_1	3.23
1.70 mg.	100 cc. NMU P_1	1.65
control	100 cc. NMU P_1	0
0.65 mg.	100 cc. NMU P_2	0.52
0.33 mg.	100 cc. NMU P_2	0.31
control	100 cc. NMU P_2	0
0.26 mg.	100 cc. NMU P_3	0.25
0.104 mg.	100 cc. NMU P_3	0.101
0.052 mg.	100 cc. NMU P_3	0.060
0.039 mg.	100 cc. NMU P_3	0.030
0.013 mg.	100 cc. NMU P_3	0.022
control	100 cc. NMU P_3	0.030

TABLE 2. COMPARISON OF PREGNANEDIOL RECOVERED FROM PREGNANCY URINE AFTER HYDROLYSIS BY HCl AND BY GLUCURONIDASE

Patient	Volume urine assayed	Pregnanediol recovered hydrolysis with	
		HCl in mg./24 hrs.	Glucuronidase in mg./24 hrs.
D. R.	100 cc.	36.8	34.2
R. A.	100 cc.	61.2	64.0
H. G.	100 cc.	72.0	73.1
M. S.	100 cc.	58.0	58.0
H. M.	100 cc.	32.0	32.6
L. M.	100 cc.	42.0	43.0
J. S.	100 cc.	44.0	45.0

nanediol as does HCl from 100 cc. pregnancy urine. Table 3 illustrates that pregnancy urine can be diluted so that 2 cc. of urine containing 0.14 mg. of pregnanediol can be readily assayed. In no case did an HCl hydrolysis effected after the glucuronidase hydrolysis yield more than the equivalent of 1 mg. pregnanediol per 24 hours. It would thus seem that in pregnancy urine at least, all of the pregnanediol is present as the glucuronide.

APPLICATION OF GLUCURONIDASE HYDROLYSIS TO STUDIES OF THE NORMAL FEMALE CYCLE

In Figure 2 is shown the level of pregnanediol excretions for a number of normal women. In these experiments assays were conducted on 500 cc. batches of urine. It will be noted that pregnanediol excretion shows a gradual increase during the follicular phase of the menstrual cycle. It does not reach its peak until a week or so before the next menses. It then falls rapidly so as to reach low levels by the time menses have begun. It will be noted that for patient K. W. the pregnanediol reached an excretion level of about 1.5 mg. per diem at the usual time but then fell abruptly, and did not rise again (to 2.5 mg. per day) until the 39th day of the cycle. As a result this girl had a 42 day cycle.

It will be noted (Fig. 2) that melting points carried out on a number

TABLE 3. EFFECT OF DILUTION OF PREGNANCY URINE (TO 100 CC. WITH H_2O) ON PREGNANEDIOL ASSAY (PATIENT D.P.).

Volume urine assayed	Theoretical, mg. pregnanediol	Actually found, mg. pregnanediol
100 cc.	6.9	6.9
50 cc.	3.5	3.5
25 cc.	1.7	1.8
10 cc.	0.69	0.75
5 cc.	0.34	0.34
2 cc.	0.14	0.14

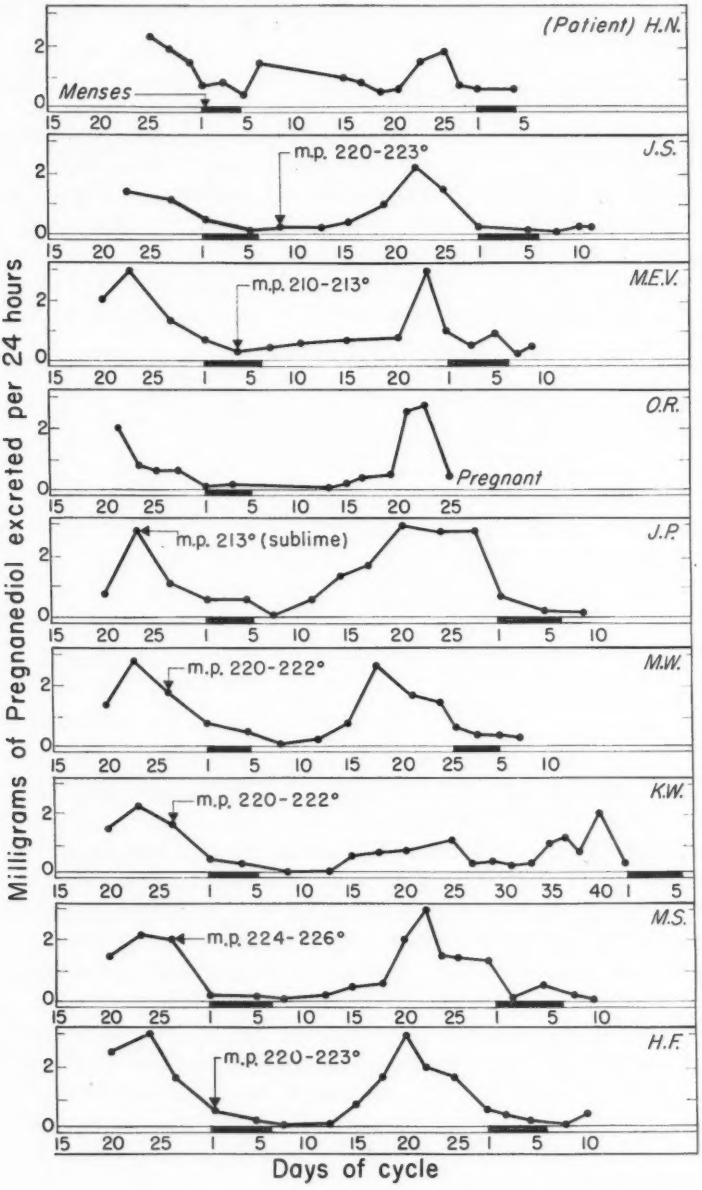

FIG. 2. The excretion of pregnanediol during the menstrual cycle.

of residues varied from 210° to 224° (pure pregnanediol melts at 236°–237°, uncorrected).[4]

THE EXCRETION OF PREGNANEDIOL DURING EARLY PREGNANCY

Guterman (1944, 1945) reported on the use of a pregnanediol test for the detection of pregnancy, a finding confirmed by one worker (McCormack 1946) and criticized by a number of workers (Semmens and McHenry, 1949; Kullander, 1950; Merivale, 1948; Reinhart and Barnes 1946). In Table 4 are shown some results which we have obtained using the glucuronidase hydrolytic procedure. Since in the normal cycling women we found no pregnanediol assay greater than 3 mg./24 hours it is of interest

TABLE 4. PREGNANEDIOL EXCRETION BY EARLY PREGNANCY CASES[1]

Patient	Approximate time lapsing since 1st missed menses	mg. pregnanediol /24 hrs.	Comment
D	1 day	3.3	
D	4 weeks	7.2	
O.R.	1 week	4.0	
O.R.	17 weeks	9.8	
A	4 weeks	5.5	
Z	4 weeks	6.5	
P	5 weeks	6.5	
E.Z.	10 weeks	15.4	
S	12 weeks	7.2	
B.H.	12 weeks	6.9	
E.H.	13 weeks	4.8	Had a previous abortion
E.H.	17 weeks	17.4	
X	14 weeks	16.1	
Y	16 weeks	4.2	Aborted 1 week later
O	18 weeks	12.5	

[1] We would like to thank Dr. I. H. Kaiser of the Department of Obstetrics and Gynecology, University of Minnesota Hospitals, for aid in collecting some of these urines.

to point out that within 1 day of the missed menses of patient D a level of 3.3 mg./24 hours and for patient R on the 5th day following the missed menses, 4.0 mg. pregnanediol was excreted. By the 4th week after the first missed menses the excretion of pregnanediol was at least 5 mg./24 hours. It is of interest to point out that patient Y, with an excretion level of only 4.2 mg. pregnanediol/24 hours in the 16th week of pregnancy aborted shortly thereafter. For patient E. H. the first urine sample collected 13 weeks after the first missed menses the pregnanediol excreted was relatively low. This patient had aborted in an earlier pregnancy and was feeling quite ill (malais and vomiting) at the time this specimen was collected. Four weeks later this had passed and the pregnanediol secretion had risen to 17.4 mg./day.

[4] The melting points were determined on a Fischer micro melting point apparatus and are uncorrected.

DISCUSSION

The use of glucuronidase as a hydrolytic means for pregnanediol in urine offers the following advantages: firstly, the conditions for effecting a complete hydrolysis with glucuronidase of NaPG results in very little hydrolysis of any other steroid glucuronide. This is true because of the use of an overnight incubation with 1,000 units of enzyme per 500 cc. urine (most conjugated steroids require an incubation of 3 to 4 days with 5,000 units per 25 cc. urine) and because the optimum pH for glucuronidase activity for NaPG is 4.5 while most of the steroid glucuronides have an optimum pH of 5.0 (Cohen, 1951). Secondly, the test is more sensitive than any other yet described, since 0.5 mg. of pregnanediol/24 hours can be accurately assayed. Apart from the time required for incubation about 6 assays can be run per working day.

Our figures for pregnanediol assays during the cycle are about one-half those reported by workers assaying for NaPG (Venning and Browne, 1937; Hain and Robertson, 1939; Buxton, 1940). This is not surprising in view of the reports by Marrian and Gough (1946) that the NaPG isolated according to the procedure of Venning and Browne (1936) contains 23% pregnaneolone. Smith and Smith (1952) have reported the presence in NaPG of up to 20% of some additional as yet not identified steroid glucuronides. Since our assay technique is a quantitative one we feel that it may offer an excellent diagnostic tool for pregnancy and for gynaecological disturbances.

SUMMARY

The glucuronidase hydrolysis has been adapted for the assay of pregnanediol in urines, by introducing a relatively simple purification procedure. This test is highly sensitive, gives material with a good melting point and offers a means of hydrolysis whereby other steroids are hydrolyzed to only a minor degree. It has been found possible to get effective assays on urines collected from girls in the early follicular phase of the cycle. It has also been found possible to utilize the assay procedure in the diagnosis of pregnancy.

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NOTES AND COMMENTS

GONADOTROPINS OF THE PITUITARY GLAND DURING INFANCY AND EARLY CHILDHOOD

The purpose of this note is to present data on the gonadotropins of the pituitary gland of 2 infants and 1 child.

I. MATERIALS AND METHODS

A. Animals.—Female Wistar rats (30 to 40 days old) were hypophysectomized, injected with pituitary homogenate and subsequently studied as described previously (Bahn and associates, 1953 a, b).

B. Pituitary Tissue.—The pituitary glands of 2 infants, 1 male and 1 female, and 1 male child, 4 years old, were obtained within three to four hours following death. No prolonged terminal illness was present in any of the 3 patients. The wet weight of the combined anterior lobes of the hypophyses of the infants was 136 mg., and that of the child, 222 mg. The effects of total doses of only 10 mg. and 30 mg. of the gland of the infants and only 5 mg. and 30 mg. of that of the child were determined owing to the limited amount and scarcity of tissue.

II. RESULTS

A. Homogenate of Infants' Hypophyses.—The data in Table 1 indicate that no follicle-stimulating hormone (FSH) and no luteinizing hormone (LH, ICSH) activity was present in 30 mg. of pituitary homogenate.

B. Homogenate of the Child's Hypophysis.—Both FSH and LH activity were detected (Table 1). Marked follicular growth accompanied by ovarian enlargement was observed at a dose of 30 mg. Although complete repair of the interstitial cells was not observed, the uterine enlargement indicated estrogen secretion and thus the presence of LH. In terms of the previously defined units, there was somewhat more than 1 unit of FSH but less than 1 unit of LH per 30 mg. of pituitary homogenate.

TABLE 1. RESPONSES TO PITUITARY HOMOGENATE

Animals		Homogenate		Ovaries		Uterus		Ovarian histology			V.O.
Condition	No.	Source	Dose, mg.	mg.	mg./gm.	mg.	mg./gm.	F.D.	C.L.	I.T.	
Hyp.	6	—	0	6.5	0.11	22.7	0.39	0	0	D	0
Intact	4	—	0	32.8	0.28	167.5	1.44	+++	+	R	+
Hyp.	2	I	10	4.1	0.07	18.6	0.30	0	0	D	0
Hyp.	2	I	30	8.1	0.10	30.6	0.36	0	0	D	0
Hyp.	4	C	5	8.7	0.12	24.6	0.33	0	0	D	0
Hyp.	2	C	30	13.4	0.18	99.9	1.31	+++	0	D	+

Abbreviations: Hyp. = Hypophysectomized; mg./gm. = Organ weight per gram body weight; D = Deficient; R = Repair; I.T. = Interstitial tissue; 0 = Absent; + = Present; V.O. = Vaginal opening; F.D. = Follicular development; I = Pooled infants' hypophyses; C = Child's hypophysis; C.L. = Corpus luteum.

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III. DISCUSSION

Functional maturity of the anterior lobe of the hypophysis is related to the growth of the gland, the production of hormone by the parenchyma, and the establishment of secretory controls. The present study is concerned primarily with the second factor, the production and storage of gonadotropins (FSH, LH) within the hypophysis.

Our previous studies showed that the anterior lobe of the pituitary gland of the adult human contained about 1 unit of both FSH and LH per 3 mg. of tissue (wet weight), or a total of approximately 100 to 200 units of each hormone. The present study indicates that the concentration and the total quantity of FSH and LH in the hypophyses of adults are much greater than those of children and those of children greater than those of infants. As compared to the adult, there was relatively less LH than FSH in the child's hypophysis. These findings are compatible with previous studies of the pituitary gland (Smith, 1939) and the urine (Nathanson and associates, 1941, and Greulich and associates, 1942) of infants and children.

The data suggest that the pituitary alterations which eventually culminate in puberty are gradual and of relatively long duration, having their onset in the first few years of life. Moreover, it appears that during childhood increases in FSH activity precede the increases in LH activity. The total quantity of gonadotropins within the gland must have risen from virtually none in infancy to between 100 and 200 units of both FSH and LH in adult life.

SUMMARY

Gonadotropic activity was virtually absent from the pooled pituitary glands of 2 infants. Both FSH and LH activity were present in the pituitary of 1 child, however, in a concentration roughly one tenth or less than that of the adult.

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1953 AWARDS AND FELLOWSHIPS

Awards and Fellowships presented by The Endocrine Society carry no obligation by the recipient to the Society or to the donors.

FELLOWSHIPS

Society Fellowships are designed to assist men or women of exceptional promise in furthering their educational training and advancement toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $5,000, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

THE AYERST, McKENNA AND HARRISON FELLOWSHIP

This Fellowship will not be awarded in 1953 in order that it may accumulate toward a $5,000 Fellowship to be awarded in 1954.

THE SCHERING FELLOWSHIP IN ENDOCRINOLOGY

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson in 1950. Dr. John D. Stoekle was chosen as the recipient of the 1951 Fellowship. Inasmuch as he was unable to accept due to a call to military service, the same Fellowship was reawarded in 1952 to Dr. C. L. Courtright. The 1953 Schering Fellowship will be in the amount of $5,000 and will henceforth be given in the same amount on alternate years.

AWARDS

THE SQUIBB INSTITUTE FOR MEDICAL RESEARCH AWARD

This Award of $1,000 was established in 1939 by The Squibb Institute for Medical Research and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr. Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; in 1950 to Dr. C. N. H. Long; and in 1951 to Dr. J. B. Collip. No Award was given in 1943. This Award was increased to the amount of $2,500 beginning with the 1952 Award and the recipient was Dr. James H. Means. A special committee of five members of the Society selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

THE CIBA AWARD

The Ciba Award, to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or pre-clinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; in 1950 to Dr. Oscar M. Hechter; and in 1951 to Dr. Albert Segaloff. Effective in 1952, the Award was increased from $1,200 to $1,800 and the recipient was Dr. Seymour Lieberman. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a Laboratory other than that in which he is at present working, it will be increased to $2,500.

BOOK REVIEW

HISTOCHEMISTRY, THEORETICAL AND APPLIED. 1953. By A. G. Everson Pearse, M.A., M.D., D.C.P. Published by Little, Brown and Co., Boston, Mass. viii & 530 pp. 4 colored plates & 109 black-and-white illustrations. Price, $12.00.

A. G. E. Pearse is a relative newcomer to the field of histochemistry. He holds an M.D. degree from the University of Cambridge and is now Lecturer in Histochemistry at the Postgraduate Medical School, University of London. One of his first contributions to histochemistry consisted of a critical review of its methodology and interpretation, written for pathologists and appearing in the British *Journal of Clinical Pathology 4:* 1 (1951). He has also introduced a number of new or modified techniques. Perhaps those having the greatest interest for endocrinologists are presented in a series of papers concerning the demonstration of the glycoprotein hormones of the anterior pituitary gland (*Nature 162:* 651, 1948; *J. Path. & Bact. 61:* 195, 1949, *64:* 791 & 811, 1952; *Stain Technol. 25:* 95, 1950).

The present volume comprises 17 chapters, beginning with a brief but fairly inclusive history of the development of this branch of science and continuing with 2 chapters on methods of fixation and sectioning, 3 on the staining of proteins, 4 on the demonstration of enzymes, 1 on the use of enzymes as histochemical reagents, and 1 each on carbohydrates, lipids, aldehydes and ketones, pigments, inorganic substances, and physical methods. The book concludes with appendices which give the detailed steps of the histochemical techniques which Dr. Pearse has himself found most useful for demonstrating various tissue constituents. Complete author and subject indices follow. A further valuable feature of the book consists of numerous black-and-white and a few colored photomicrographs illustrating the appearance of sections prepared by some of the histochemical methods discussed.

A rash of books devoted entirely or largely to histochemical techniques has appeared in the last few years. Outstanding are the treatises by Lillie (1948), Romeis (1948, 15th ed., German), Glick (1949), Gatenby and Beams (1950, 11th ed.), Gomori (1952), and Lison (1953, 2nd ed., French). In this reviewer's opinion, the new Pearse bids fair to become the leader, even amongst so notable a collection. In each chapter, Dr. Pearse reviews the chemistry and biological importance of the substances in question and then goes on to discuss critically the methods used for their demonstration and differentiation. The bibliographic references are numerous and appear to include most of the major contributions in each area.

Pearse has omitted some references which appear fundamental, for example, the definitive experimental paper and review by Marcus Singer concerning the nature of acidic and basic staining of proteins (*J. Biol. Chem. 75:* 133, 1948; *Internat. Rev. Cytol. 1:* 211, 1952). Likewise, the techniques which he has selected to give in detail represent his particular preferences, whereas numerous methods which are possibly equally satisfactory are barely mentioned. While Pearse's omissions in this respect are not serious, the usefulness of some methods which he has overlooked should not be lost sight of.

Needless to say, in a field which is growing so rapidly and to which several journals are now entirely devoted, no book can long remain up-to-date.

While the organization of this book does not permit any special emphasis on the endocrine glands, it must be apparent that modern endocrine histophysiology depends increasingly on histochemical techniques. Pearse's *Histochemistry* should prove a valuable addition to the library of any endocrinologist who intends either to apply histochemical methods or to interpret the significance of his own or others' results.

HELEN WENDLER DEANE